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# The evolutionary roots of reproductive ageing and reproductive health across the tree of life

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Jean-Francois Lemaitre and Jean-Michel Gaillard

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# The evolutionary roots of reproductive ageing and reproductive health across the tree of life

**Topic editors**

Jean-Francois Lemaitre — UMR5558 Biométrie et Biologie Evolutive (LBBE), France

Jean-Michel Gaillard — Université Claude Bernard Lyon 1, France

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Jordi Figuerola,
Spanish National Research Council (CSIC), Spain

*CORRESPONDENCE
Jean-François Lemaître
jean-francois.lemaitre@univ-lyon1.fr

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# Editorial: The evolutionary roots of reproductive ageing and reproductive health across the tree of life

Jean-François Lemaître* and Jean-Michel Gaillard

Univ Lyon, Université Lyon 1, CNRS, Laboratoire de Biométrie et Biologie Évolutive UMR 5558, Villeurbanne, France

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Editorial on the Research Topic
The evolutionary roots of reproductive ageing and reproductive health across the tree of life

## Introduction

The last decades have seen a burst in the number of empirical studies documenting reproductive ageing (i.e. the decrease in reproductive performance with increasing age; also called reproductive senescence) under very diverse environmental conditions (e.g. in wild and captive populations). So far, most detailed investigations (and subsequent empirical evidence) of reproductive ageing patterns have been conducted on birds and mammals (Holmes et al., 2003; Nussey et al., 2013; Lemaître et al., 2020; Comizzoli and Ottinger, 2021), which is easily explained by the fact that, for a long time, most long-term individual-based longitudinal studies of populations in the wild focused on these Vertebrate classes (Clutton-Brock and Sheldon, 2010). However, empirical studies on other taxonomic groups have started to emerge, enabling us to broaden our understanding of the eco-evolutionary roots of reproductive ageing across the tree of life (see e.g. Crosland et al.; English et al.; Tully for three articles from this Research Topic documenting reproductive ageing in invertebrates, see next sections for details).

Reproductive ageing is often assumed to be negligible in species with indeterminate growth, because the increasing size of females throughout the entire life is expected to be associated with an increased fecundity (Jones and Vaupel, 2017). However, increased fecundity with age does not preclude ageing in other reproductive traits. In particular, a decrease in offspring performance with maternal age might counter-balance the fecundity increase and lead to lower reproductive success with increasing age (see Depeux et al., 2020 for a case study in the common pill woodlouse, *Armadillidium vulgare*). To date, empirical support for an absence of reproductive ageing in indeterminate growers is badly missing. In this Research Topic, Vrtílek et al. shed light on reproductive ageing patterns in indeterminate growers by compiling an impressive set of published case-studies on ray-finned fishes (*Actinopterygii*). They found evidence of reproductive ageing in 26% of studies, a figure that is likely

underestimated due to the short age range relative to reproductive lifespan that was covered in most studies. Reproductive ageing occurs in both sexes in a wide range of reproductive traits including breeding frequency and sperm quality. This study suggests that albeit variable among taxa, reproductive ageing does occur in indeterminate growers and is therefore likely more pervasive across the tree of life than generally assumed.

Despite this consistently increasing set of species displaying reproductive ageing, the eco-evolutionary causes and consequences of this process in terms of population dynamics and reproductive health remain to be fully deciphered. This Research Topic therefore brings together contributions whose common aim is to address the complexity of reproductive ageing through the lens of evolutionary biology, while using very different approaches, such as laboratory experiments, comparative analyses of ageing patterns across species, or the tracking of individual age-specific reproductive trajectories within populations in the wild.

## Reproductive ageing and evolutionary theories of ageing

All evolutionary theories of ageing are rooted in the decrease of the force of selection when organisms age, leading to expect ageing to occur in all biological functions. However, most theoretical work has focused on the rate and shape of actuarial ageing (i.e. the increase in mortality risk with increasing age). For instance, the landmark work by Hamilton (1966) provides clear predictions for actuarial ageing and only briefly discuss reproductive ageing, stating that "*the problem seems to be biologically more complex than that concerning the mortalities*" (p. 42). This might explain why reproductive ageing has been much less studied than actuarial ageing until recently. The diversity of patterns even within a single taxonomic group (see e.g. Lemaître et al. (2020) for a comparative analysis across mammals) and the quite large number of traits involved in the sequential process of reproduction (Lemaître and Gaillard, 2017) support Hamilton's view of the complexity of reproductive ageing. In this Research Topic, Lee and Chu, 2023 partly fill this knowledge gap by proposing an optimal life history model of ageing in fertility. Despite its simplicity, this model nicely captures the diversity of ageing patterns in fertility and shows that the life history strategies and the life styles, likely in an interplay with the environmental context, influence both the direction and the shape of fertility changes over age during the adulthood stage. The awareness that the environmental context markedly influences ageing patterns is growing, but we still lack a mechanistic understanding on how this might happen. Crosland et al.'s experimental work provides a highly relevant case study in that context using the mealworm beetle (*Tenebrio molitor*). They raised beetles under environments with highly contrasting quality by manipulating relative humidity conditions. They found that females raised in good conditions (i.e. high relative humidity) grew faster, lived longer, and produced more offspring over their lifetime than females raised in poor conditions (i.e. low relative humidity). On the other hand, females raised in good conditions displayed a stronger reproductive ageing than females raised in poor conditions, providing a direct support for evolutionary theories predicting a trade-off between fast growth and high fecundity early in life and performance of fitness-related traits (reproductive output in that case) late in life (e.g. disposable soma theory, see Kirkwood and Rose, 1991).

## Ageing in male's fertilization efficiency

Compared to females, our current knowledge on male reproductive ageing is limited, especially regarding the effects of age on the efficiency at fertilizing eggs. Yet, male's Darwinian fitness is often predominantly determined by fertilization success and one could thus easily predict that reproductive ageing in males should be primarily driven by a decline in the performance of ejaculate-related traits, such as sperm quality or sperm quantity, with increasing age. The ageing in male's fertilization efficiency is nonetheless attracting lots of interest in public health studies, as the age at which fathers conceive offspring is continuously delayed in western societies. The wave of studies performed in that context have demonstrated that both sperm quality (e.g. sperm motility) and quantity (e.g. semen volume) show unambiguous evidence of ageing across human populations (Johnson et al., 2015). It is only recently that evolutionary biologists have started to investigate ageing in ejaculate-related traits. Yet, this process could have major consequences in terms of eco-evolutionary dynamics (Bonduriansky et al., 2008), especially in polyandrous or promiscuous species where sperm competition occurs (i.e. when ejaculates from different males compete to fertilize one or more oocytes, see Parker, 1970). So far, no consensus on the pervasive nature of ageing in ejaculate-related traits has been reached, simply because most of the studies performed in that context failed to encompass the full male reproductive life, which ultimately impact the likelihood to detect ageing (Sanghvi et al., 2023). Two empirical studies published in this Research Topic, both performed on birds, thoroughly embrace this topic (Meunier et al.; Míčková et al.). A third (Fricke et al.), very complementary to the previous ones, focus on the putatively widespread ageing of the seminal fluid content (i.e. non-fertilizing part of the ejaculate).

Using artificial insemination experiments in North African houbara bustard (*Chlamydotis undulata undulata*), Meunier et al. disentangle the influence of pre-meiotic ageing (i.e. effects due to male's age) and post-meiotic ageing (i.e. effects due to sperm's age) on diverse sperm features and reproductive success metrics. They document a marked decline in both quantity (i.e. ejaculate volume, sperm concentration) and quality (i.e. motility, velocity) with increasing male's age, which ultimately translate into the hatching of chicks displaying lower growth rates and higher mortality rate. Unexpectedly, the sperm age has a positive influence on both sperm quality traits and hatching success.

Focusing on pre-meiotic sperm ageing in a population of barn swallow (*Hirundo rustica rustica*) intensively monitored during a longitudinal study, Míčková et al. provide a thorough analysis of the age-specific changes in diverse sperm characteristics over the male's life course. While their analyses reveal a decline in sperm length

with increasing age, both sperm velocity and sperm production intensity (assessed by the size of the cloacal protuberance) show no sign of ageing. Interestingly, the authors also document a negative association between the size of the cloacal protuberance and lifespan occurs among males, suggesting potential survival costs of maintaining high levels of sperm production throughout the life.

Finally, Fricke et al. provide the first qualitative review of the effect of male's age on seminal fluid content. Indeed, male's reproductive success is also mediated by the non-fertilizing part of the ejaculates (Perry et al., 2013), especially by the seminal fluid proteins produced by the accessory reproductive glands. These proteins typically interact with sperm cells or modulate female's reproductive behavior to increase male's fertilization success (e.g. Chapman et al., 2003). Everything else being equal, any decline in the quality of the seminal fluid should impair male's reproductive success. Fricke et al. compile fourteen papers reporting an effect of male's age on seminal fluid proteins. Their review highlights contrasted patterns among studies (e.g. from a decrease to an increase in the concentration of some proteins) possibly caused by major methodological issues such as a too short age range covered relative to reproductive lifespan (see also above) or an absence of control for prior mating experiences.

## The asynchrony between reproductive and non-reproductive ageing

One salient challenge currently associated with the study of reproductive ageing is the need to embrace the sequential nature of reproduction. In both sexes, reproductive success (i.e. the number of offspring produced by a female and alive at the end of the parental care period) involves a sequence of events (e.g. implantation success, birth success in females, pre- and post-copulatory competition in males) underpinned by phenotypic traits (size of secondary sexual traits) and physiological functions (e.g. milk production, spermatogenesis) that can all decline with increasing age (Lemaître and Gaillard, 2017). Similar observations have been made for other phenotypic traits (e.g. physiological or behavioural traits) - not directly linked with reproduction - that all seem to follow their own age-specific trajectory (Promislow et al., 2006; Gaillard and Lemaître, 2017). However, despite theoretical predictions (Williams, 1957; Maynard Smith, 1962; Moorad and Ravindran, 2022), the magnitude of the asynchrony in ageing among reproductive traits (and beyond) is yet to be quantified and the role of natural selection in shaping this mosaic pattern of ageing remains to be empirically tested. Two articles published in this Research Topic directly address these questions (Moullec et al.; Tully).

Taking advantage of the long-term studies of two Alpine swift (*Tachymarptis melba*) colonies, Moullec et al. performed a thorough analysis of ageing patterns in both sexes across eleven phenotypic and life history traits, including notably four reproductive traits (i.e. laying date, clutch size, brood size at hatching and at fledgling). Their analyses highlight marked differences in age-specific patterns among traits and between sexes. For instance, among females, tail length shows a late age at the onset of ageing (ca. 15 years of age) while the brood size at hatching starts to decline earlier (ca. 12 years of age), but at a much lower rate. These findings indicate differences between reproductive and somatic ageing. Interestingly, when focusing strictly on reproductive traits, ageing is detected only in brood size at hatching and at fledgling and only in males, an intriguing result further discussed in the context of sex-differences in life history tactics.

Differences between actuarial and reproductive ageing patterns are also at the core of the study by Tully focusing on Collembola (*Folsomia candida*) lineages originating from two phylogenetically distinct clades. This study reveals a clear decline in clutch size with increasing age, with quite marked differences in both onset and rate of reproductive ageing among clades and experimental treatments. However, for most clades, the onset of reproductive ageing in clutch size is earlier than the onset of actuarial senescence. On the other hand, ageing in egg's size seems to be negligible, a result consistent across all lineages. In addition, Collembola under a dietary restriction regime show a delayed onset of reproductive ageing, as well as a lower rate of reproductive ageing, a finding discussed at the light of recent advances in the study of phenotypic plasticity in ageing.

## The influence of environmental conditions on reproductive ageing

There is increasing evidence that the intensity of ageing is influenced by either current or past environmental conditions. For instance, mammals from species with a short generation time display a delayed and weaker actuarial ageing in zoos than in the wild, leading captive individuals to outlive markedly their conspecifics in the wild (Tidière et al., 2016). On the other hand, poor early-life conditions did not have detectable effects on actuarial ageing from the meta-analysis performed by Cooper and Kruuk (2018). The figure for reproductive ageing seems to be different although the influence of current conditions on reproductive ageing has been less investigated on reproductive ageing than on actuarial ageing. In this Research Topic, two studies provide clear evidence for an influence of current environmental conditions on reproductive ageing. Naciri et al. perform a detailed analysis of reproductive ageing in polar bears (*Ursus maritimus*) in the intensively monitored population of Svalbard. They provide clear evidence of reproductive ageing in both litter production and litter size, which decline slightly for females aged 15-20 years and markedly from 20 years of age onwards. On the other hand, neither offspring mass or survival within the maternal care period display any evidence of reproductive ageing. Although the response of reproductive ageing metrics to variation in environmental conditions is not investigated, the authors provide clear evidence that older females are more susceptible to environmental harshness (measured by the date of sea-ice break-up) than prime-aged ones, suggesting that ageing is more pronounced under hash environmental conditions. Likewise, Kappeler et al. compare reproductive ageing between wild and captive conditions in

two lemur taxa (Verreaux's sifakas, *Propithecus verreauxi* and redfronted lemurs, *Eulemur rufifrons*). They provide consistent evidence of reproductive ageing (although not statistically significant for one population in the wild likely because of a lack of statistical power), supporting again the view that reproductive ageing is the rule rather than the exception in mammals (Lemaître et al., 2020). The intensity of reproductive ageing differs between captive and wild conditions. Interestingly, the decrease of reproductive performance with increasing age is delayed and less steep in the wild than under captive conditions, while the opposite generally occurs for actuarial senescence (Tidière et al., 2016). This discrepancy likely involves husbandry decisions as mating patterns are generally controlled by managers in captive populations of large mammals. These findings provide new evidence for variable ageing patterns within a same species in response to different environmental contexts.

Previous studies indicate that early-life conditions seem to be influential on the intensity of reproductive ageing. Thus, in their meta-analysis, Cooper & Kruuk (2018) provided evidence of early-life effects on reproductive ageing (see also Nussey et al., 2007 for a convincing example in red deer, *Cervus elaphus*). Two case studies in this Research Topic test whether early-life conditions shape reproductive ageing. Taking advantage from the long-term monitoring of male antlers in two roe deer (*Capreolus capreolus*) populations facing with markedly different environmental conditions, Cambreling et al. document a decrease of antler size with increasing age from 7 years of age onwards irrespective of the population, which supports the occurrence of reproductive ageing in males. Moreover, in both populations, ageing in antler length is delayed for males born in good cohorts (i.e. heavy when 8 months of age) compared to those born in poor cohorts (i.e. light when 8 months of age), which supports the influence of early-life conditions on reproductive ageing and indicates that male antler size is a honest signal of phenotypic quality. From an experimental approach, Vedder et al. assess the influence of early-life conditions on performance later in life using a captive population of Japanese quail (*Coturnyx japonica*). They used the incubation temperature (normal vs. cool) to mimic variation in the quality of early-life conditions. The reproductive performance (measured by the daily laying rate) declines markedly with increasing female age, supporting reproductive ageing. However, early-life conditions do not have any detectable influence on reproductive ageing, and more generally on any adult life history trait. Although these findings might suggest that incubation temperature is not an important driver of early-life conditions, they are also in line with the general observation that mild environments such as that provided for captive female quails in this study decrease the intensity of reproductive ageing, and thereby the magnitude of silver spoon (sensu Grafen, 1988) or carry-over (see Harrison et al., 2011) effects.

## From reproductive ageing to reproductive health

A substantial variation in both the onset and the rate of reproductive ageing has been reported within species (Gaillard and Lemaître, 2020) and the multiple pathologies that can impact the reproductive machinery could potentially contribute to the pronounced reproductive ageing observed in some males and females. However, the influence of past and current reproductive diseases on reproductive ageing has almost never been quantified and the eco-evolutionary roots of such diseases are yet to be fully deciphered (but see Alvarado, 2013; Jasienska, 2013). Similarly, it is largely unknown to what extent the risk of diseases (and in particular, reproductive diseases) has, in a given environment, influenced the evolution of age-specific reproductive tactics. Three articles from this Research Topic shed a new light on these questions.

In a perspective paper, Dujon et al.explore the untapped associations between age-specific reproductive allocation (including reproductive ageing) and cancer risk. These authors first provide an overview of the possible evolutionary trade-offs linking age-specific fertility and cancer risk, by notably focusing on genes with pleiotropic and antagonistic effects. They go beyond the classic examples of *Xmrk* melanoma-promoting oncogene in fish or the more controversial BRCA1 and BRCA2 alleles in humans by questioning the potential pivotal role of genes controlling gamete production in the context of cancer risk. Then, they review the multiple and complex relationships that putatively link the allocation of resources towards sexual competition and reproduction over the life course to the evolution of cancer-defense mechanisms and elaborate on the selective forces played by cancer risk on the evolution of reproductive ageing.

In line with the review from Dujon et al., Bieuville et al. further explore the association between women's reproductive history and post-menopausal health by investigating the life history determinants of breast cancer risk. More specifically, they reveal that postmenopausal breast cancer risk is higher in the subset of women who experienced the highest number of lifetime menses. In addition, an elevated number of menses also appears to be associated with an earlier onset of breast cancer after menopause. These results are discussed in the context of an evolutionary mismatch where the rapid increase in number of menses through women's recent evolutionary history is responsible for a higher lifetime exposure to cyclic reproductive hormones (e.g. progesterone), which triggers the risk of developing breast cancer. This study thus emphasizes the tight link between age-specific reproductive effort and reproductive health and calls for further work testing how the association between reproductive effort in early life and reproductive ageing (e.g. Nussey et al., 2006) might be mediated by reproductive health issues.

Finally, English et al. extend the range of reproductive traits that are traditionally analyzed in reproductive ageing studies by focusing on a key public health issue: the abortion rate. Using the long-lived tsetse fly (*Glossina morsitans morsitans*) as a biological model, they document a higher rate of abortion in old females compared to middle-age females at both late-larval stage and egg-stage. Importantly, this increase in reproductive loss in late life does not appear to be adaptive as it does not reduce the interval of time before the next laying event nor increase the body mass of the future offspring. This study further demonstrates that abortion rates increase under harsh environment and thus calls for additional studies investigating both the occurrence and the magnitude of this

reproductive process, largely neglected in the evolutionary biology literature.

## Concluding remarks: exploring the genetic and physiological basis of reproductive ageing

The content of this Research Topic clearly demonstrates the lively nature of the current research performed in the field of reproductive ageing. Moreover, it highlights that deciphering the eco-evolutionary roots of reproductive ageing as well as its consequences in terms of both population dynamics and public health require inputs from many research areas. It is also important to emphasize that the topic explored by the various contributions included in this Research Topic are only the tip of the iceberg, and other salient research questions on reproductive ageing need to be tackled in the near future. Among them, we can notably mention the quantification of the amount of individual heterogeneity in both the onset and rate of reproductive ageing but also the identification of the physiological and genetic markers of reproductive ageing in wild animal populations. Regarding the latter, the study from Meyer et al. published in this Research Topic paves the road for such research projects. Here, taking advantage of a long-term individual based study in common tern (*Sterna hirundo*), these authors explore the age-related changes in autosomal methylation for both males and females in this population. The key role that epigenetic changes (hyper- or hypo- methylation) plays on ageing is currently attracting lots of interest (e.g. Lu et al., 2023) and their analyses reveal that autosomal methylation levels decline with increasing age in females, but not in males. However, as emphasized by Meyer et al. senescence patterns do not differ between sexes in common terns, which highlight that the association between age-specific changes in epigenetic profiles and ageing, notably reproductive ageing, is complex and requires further investigations.

## Author contributions

JL: Conceptualization, Writing – original draft, Writing – review & editing. JG: Conceptualization, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The Effect of Manipulated Prenatal Conditions on Growth, Survival, and Reproduction Throughout the Complete Life Course of a Precocial Bird

*Oscar Vedder[1]*, Coraline Bichet[1,2] and Barbara Tschirren[3]*

*[1] Institute of Avian Research, Wilhelmshaven, Germany, [2] Centre d'Etudes Biologiques de Chizé, CNRS-La Rochelle Université, UMR-7372, Villiers-en-Bois, France, [3] Centre for Ecology and Conservation, University of Exeter, Penryn, United Kingdom*

The quality of the environment individuals experience during development is commonly regarded as very influential on performance in later life. However, studies that have experimentally manipulated the early-life environment and subsequently measured individual performance in all components of fitness over the complete life course are scarce. In this study, we incubated fertile eggs of Japanese quail (*Coturnix japonica*) at substandard and standard incubation temperature, and monitored growth, survival, and reproduction throughout the complete life course. While embryonic development was slower and hatching success tended to be lower under substandard incubation temperature, the prenatal treatment had no effect on post-hatching growth, survival to sexual maturity, or age at first reproduction. In adulthood, body mass and investment in individual egg mass peaked at middle age, irrespective of the prenatal treatment. Individual reproduction rate declined soon after its onset, and was higher in females that lived longer. Yet, reproduction, and its senescence, were independent of the prenatal treatment. Similarly, adult survival over the complete lifespan was not affected. Hence, we did not find evidence for effects on performance beyond the developmental period that was manipulated. Our results suggest that effects of unfavorable developmental conditions on individual performance later in life could be negligible in some circumstances.

**Keywords: aging, birds, developmental conditions, incubation, lifespan, lifetime reproductive success, senescence, silver spoon effect**

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****Correspondence:***
*Oscar Vedder*
*oscar.vedder@ifv-vogelwarte.de*

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## INTRODUCTION

Quality differences in the environment individuals experience during development may have long-term effects on phenotype, survival, and reproduction, thereby profoundly impacting individual life histories and fitness (e.g., Kruuk et al., 1999; Metcalfe and Monaghan, 2001; Lummaa and Clutton-Brock, 2002; Reid et al., 2003; van de Pol et al., 2006; Nussey et al., 2007; Hamel et al., 2009; Hayward et al., 2013; Spagopoulou et al., 2020). Yet, whether negative fitness consequences of a poor developmental environment are universal, and at what life stage they are primarily incurred, remains difficult to infer (Cooper and Kruuk, 2018; Vedder et al., 2021). This difficulty partly

stems from the scarcity of studies that combined experimental manipulation of the developmental environment with complete monitoring of individual lifelong performance. While experimental manipulation is required to establish an effect of the developmental environment *per se*, complete monitoring is required to avoid the problem of "the invisible fraction" (Grafen, 1988). This term refers to the fraction of individuals that does not survive, or recruit, to the pool of individuals of which fitness is measured. This fraction can be substantial due to mortality during individual development, or dispersal, and, if this fraction is non-random, can bias conclusions regarding the importance of the environment during development for complete fitness and life histories (Mojica and Kelly, 2010; Goodrich and Roach, 2013; Garratt et al., 2015; Vedder et al., 2021).

In this study, we experimentally manipulated the quality of the environment during early development in Japanese quail (*Coturnix japonica*), and monitored all age-specific components of fitness that compose total fitness: embryonic survival, survival to reproduction, age at onset of reproduction, lifelong age-specific reproduction and adult survival. Because effects of developmental conditions on fitness may be mediated by body mass differences in the wild (Plard et al., 2015; Ronget et al., 2018), we also monitored juvenile growth and adult body mass throughout life. Japanese quail are a popular avian model for research on physiology and senescence (Ottinger, 2001), in which all abovementioned components of fitness can be quantified. Moreover, it exhibits a very fast life history with a lifespan that rarely exceeds 3 years (Woodward and Abplanalp, 1971). To manipulate the quality of the early developmental environment, we artificially incubated fertile eggs at different incubation temperatures.

Low incubation temperatures in birds generally lead to slower embryonic development and reduced hatching success (Decuypere and Michels, 1992; DuRant et al., 2013). In addition, effects of incubation temperature on post-hatching phenotype are frequently reported, which may carry through to adulthood (DuRant et al., 2013). For example, in wood ducks (*Aix sponsa*) a low incubation temperature led to slower post-hatching growth, increased corticosterone levels, poorer locomoter performance, and a lower probability of recruitment to the breeding population (DuRant et al., 2010; Hopkins et al., 2011; Hepp and Kennamer, 2012). In blue tits (*Cyanistes caeruleus*) a low incubation temperature increased resting metabolic rate in the nestling stage (Nord and Nilsson, 2011), but intermediate incubation temperatures were associated with the highest adult body mass in the 3% of nestlings that were sampled in adulthood (Nord and Nilsson, 2016). An experiment in captive zebra finches (*Taeniopygia guttata*) found lower survival in adulthood in response to low incubation temperature, with survival being monitored until 77% of hatchlings were no longer alive (Berntsen and Bech, 2016). Yet, this does not necessarily imply that lower incubation temperatures negatively affect total fitness. Indeed, in common terns (*Sterna hirundo*) and Japanese quail a lower incubation temperature reduced telomere attrition during embryonic development (Vedder et al., 2018; Stier et al., 2020). Since increased telomere attrition may mechanistically link early-life adversity to decreased survival later in life-life (e.g., Heidinger et al., 2012; Eastwood et al., 2019), this illustrates the need to measure performance during an individual's entire life.

In this study, we randomly assigned fertile Japanese quail eggs to incubation at standard and substandard temperature, and subsequently monitored individual performance in terms of growth, reproduction, and survival. We specifically monitored adult performance of females over their complete lifespan, to also test for senescence effects that only occur late in life. To our knowledge, this represents the most detailed study on the effects of incubation temperature on lifelong performance in any bird species to date.

# MATERIALS AND METHODS

## Experimental Procedures and Data Collection

The study was performed in 2018 in a captive population of Japanese quail maintained at the Institute of Avian Research in Wilhelmshaven, Germany. In four breeding rounds, a total of 96 pairs were housed in breeding cages (122 cm × 50 cm × 50 cm). All individuals were of similar age (1 year old). They spent 8 days together in the breeding cages, and eggs were collected at a daily basis. Eggs were marked with an indelible marker and weighed, to the nearest 0.01 g, at the day of collection. Females were isolated from males, at least 10 days prior to pairing, to prevent stored sperm of previous males to fertilize any eggs (see Birkhead and Fletcher, 1994). The collected eggs were stored at 12°C and artificially incubated in four rounds, always within 7 days of laying. Excluding the egg that was laid on the first day after pairing (which was always undeveloped) for each pair, we collected a total of 604 eggs.

For the first 7 days, incubation of these eggs was performed at 37.7°C and 50% relative humidity in two identical fully automatic incubators (Grumbach, ProCon automatic systems GmbH & Co., KG, Mücke, Germany) that turned eggs every hour. After 7 days of incubation, eggs were candled. 525 of 604 eggs (87%) from 93 parent pairs contained a live embryo, of which 263 randomly selected eggs were further incubated at 37.7°C, whereas the other 262 eggs containing an embryo were further incubated at 36.0°C. The specific temperatures of the experimental groups were chosen as 37.7°C represents a standard incubation temperature that maximizes hatching success (Stier et al., 2020), while 36.0°C represents a considerable reduction in temperature that still results in viable hatchlings (Ben-Ezra and Burness, 2017). There was no difference in fresh egg mass between the two treatment groups (mean = 11.64 g for both groups, $\chi^2 = 0.35$, $\Delta df = 1$, $P = 0.552$). The subsequent incubation was performed in the same identical incubators as in the first week, but with one incubator set to 36.0°C. The incubator with the lower incubation temperature was alternated between rounds. After 14 days of incubation, the eggs were placed in marked individual compartments to allow the hatchling to be linked to the egg it hatched from. Further incubation until hatching was done at the same temperatures, but with 70% relative humidity and no egg turning, in two hatching incubators

(Favorit, HEKA Brutgeräte, Rietberg, Germany). From 15 days of incubation onward, both hatching incubators were checked every 6 h to monitor hatching until no viable embryos were left (after around 20 days of incubation). This allowed us to measure the total incubation duration that each hatchling required with a resolution of 0.25 days.

Once a day, all chicks that had hatched in the previous 24 h were taken out of the incubators, marked with a numbered plastic leg ring, weighed to the nearest 0.01 g, and placed in heated rearing cages (109 × 57 × 25 cm, Kükenaufzuchtbox Nr 4002/C, HEKA Brutgeräte, Rietberg, Germany), with maximally 30 individuals of mixed incubation treatment per cage. In the rearing cages, water and food were provided *ad libitum*, with a 16-8 h light-dark cycle. The rearing diet contained 21.0% protein and had a caloric value of 11.4 MJ/Kg (GoldDott, DERBY Spezialfutter GmbH, Münster, Germany). The temperature of the rearing cages was set at 37.0°C at hatching and gradually lowered to room temperature (20-25°C) over the course of two weeks. After 14 days, the plastic leg rings were replaced with uniquely numbered aluminum rings, and the chicks transferred to outdoor aviaries in mixed treatment groups. Here, they were kept on an adult diet with 19.0% protein and a caloric value of 9.8 MJ/Kg (GoldDott, DERBY Spezialfutter GmbH, Münster, Germany), and received a minimum of 16 h of light per day.

All chicks were weighed at an age of 7, 14, 28, and 42 days after individual hatching, to the nearest 0.01 g at age 7 d, and to the nearest g at all later ages. Chicks were sexed based on plumage characteristics, which are distinctly different between males and females from the age of 4 weeks. All chicks that died prematurely were molecularly sexed (following Becker and Wink, 2003), except one. From 5 weeks onward, the chicks were individually checked for the onset of reproductive activity every 2-3 days. For males this was done by checking for the production of cloacal foam, which is a good indicator for sexual activity (Sachs, 1969). For females this was done by checking for the presence of an egg in the oviduct, which can be easily established by palpation. Each individual was weighed again at its age of onset of reproduction (hereafter referred to as "age at sexual maturity"). At the onset of reproduction, each female was temporarily housed in a breeding cage until she laid at least 4 eggs. This way, her laying rate was established and her eggs were weighed to the nearest 0.01 g on the day of laying. Afterward, the females were moved back into the outdoor aviaries, with a minimum of 16 h of light per day to ensure the continuation of reproductive activity (Kovach, 1974). All mortality was monitored on a daily basis, and all females were subsequently weighed and monitored for reproductive investment (laying rate and egg mass across 7 days) at approximately 0.5 year intervals. Due to logistic challenges of keeping large numbers of males, and the difficulty of testing individual male reproductive performance independent of partner performance, we did not monitor male adult performance.

## Statistical Analyses

The effect of the treatment (as a categorical fixed effect) on hatching success, and post-hatching survival to sexual maturity, were analyzed using generalized linear mixed models with a binominal error distribution and a logit link function. Treatment effects on incubation duration, body mass at hatching and subsequent growth (i.e. mass at day 7, day 14, day 28, day 42, and sexual maturity), and age at sexual maturity, were analyzed using linear mixed models with normal error distributions. In the model for body mass growth, age was treated as a categorical fixed effect, such that the effect of the treatment on body mass was tested at all ages in a single model (i.e., the interaction effect between treatment and age in **Table 1**). All models included parent pair identity as a random effect, and the model for body mass growth additionally included individual identity, nested within parent pair, as a random effect. For the analyses on growth, post-hatching survival to sexual maturity, and age at sexual maturity, sex (and its interaction with age) was included as a fixed effect to account for potential sex differences in development and survival to sexual maturity.

The adult females were, on average, weighed and monitored for reproductive performance (see above) at an age of 58, 243, 404, 591, 863, 1,012, 1,134, and 1,224 days, if they reached that age. Effects of the incubation treatment on adult body mass, laying rate, and egg mass, were analyzed together with age (in days) as a continuous variable. To account for non-linear effects of age, we also always added the quadratic term of age. We also included age at last sampling per individual (following van de Pol and Verhulst, 2006) to assure that the effect of age represents the within-individual pattern, unconfounded by individuals with non-random phenotypes (with respect to the dependent variable) not surviving to later sampling ages. To test whether the incubation treatment had an effect on the rate of senescence (i.e., a within-individual decline in performance with age) we also tested for the interaction between treatment and age. These models included parent pair identity, and individual identity (nested within parent pair) as random effects. The models for adult body mass and egg mass assumed a normal error distribution, and the model for laying rate a binominal error distribution with a logit link function. All aforementioned models were run in MLwiN (version 2.22; Rasbash et al., 2004) and significance ($P < 0.05$) was assessed using the Wald statistic.

Breakpoint models may better describe non-linear effects of age, can pinpoint the age of peak performance, and assess whether a decline in performance (senescence) occurs after the peak (e.g., Berman et al., 2009). However, in our case, they have the disadvantage that age should be treated as a discrete variable. We therefore repeated the analyses in which a trait peaked at middle age with breakpoint models, for which we binned our continuous age data to discrete ages representing the average age of all individuals in the 8 abovementioned measurement periods (see **Supplementary Information**).

We tested whether adult survival depended on the incubation treatment with a mixed-effects Cox model (Ripatti and Palmgren, 2000; Therneau et al., 2003; Nenko et al., 2018). This model included the treatment as explanatory variable and survival as response variable using the "coxme" function in the "coxme" R package (Therneau, 2018) implemented in R 3.5.3 (R Core Team, 2014). The identity of the parent pair was added as a

random effect. The data were encoded with a zero as starting point for all individuals and with the lifespan until death (in days, max. = 1365, $n = 180$) as stop (Therneau, 2018).

# RESULTS

## Early-Life Performance

Hatching success tended to decrease with incubation temperature, from 77.6% at 37.7°C to 70.2% at 36.0°C ($\chi^2 = 3.60$, $\Delta df = 1$, $P = 0.057$). Lower temperature also delayed hatching, by roughly one day, due to longer development time (18.23 days vs. 17.16 days, coefficient ± SE = 1.07 ± 0.04 d, $\chi^2 = 626.12$, $\Delta df = 1$, $P < 0.001$).

There was no evidence for body mass at hatching and subsequent growth to be affected by the incubation treatment (**Figure 1** and **Table 1**). 96.6% of hatchlings survived to sexual maturity, and there was no indication for hatchling survival to be different between the incubation treatments (**Table 2**), nor was the age at sexual maturity different between the incubation treatments (**Figure 1** and **Table 2**).

## Adult Performance

Adult body mass peaked at middle age, as evidenced by a quadratic effect of age (**Figure 2** and **Table 3**). Breakpoint modeling indicated this peak to occur around 404 days of age, after which body mass declined (**Supplementary Figure 1A** and **Supplementary Table 1A**). There was, however, no effect of the incubation treatment on adult body mass, or on how it changed with age (**Figure 2** and **Table 3**). There was also no selective mortality in relation to body mass, as the last sampling age to which individuals survived was not related to body mass (**Table 3**).

There was rapid senescence in reproductive performance, with laying rate strongly declining with age within individual females (**Figure 3** and **Table 4**). Females that survived to later sampling ages had overall better reproductive performance (**Figure 3** and **Table 4**), indicating selective mortality of poor layers. There was no evidence for the incubation treatment to have an effect on laying rate, or the change in laying rate with age (**Figure 3** and **Table 4**). Investment in individual egg mass peaked at middle age, but also was not affected by the incubation treatment, or related to the last sampling age to which individuals survived (**Figure 4** and **Table 5**). Breakpoint modeling indicated the peak in individual egg mass to occur around 243 days of age, after which there was relatively little change with age (**Supplementary Figure 1B** and **Supplementary Table 1B**).

Adult survival did not differ between the incubation treatments (**Figure 5**, Hazard ratio ± SE = 0.94 ± 0.18, $Z$-value = −0.36, $P = 0.720$).

**TABLE 1 |** Summary of a linear mixed model testing for effects of incubation treatment on age- and sex-specific body mass in Japanese quail chicks.

| **Dependent variable: body mass (g)** | | | | |
|---|---|---|---|---|
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 8.40 (1.23) | | | |
| Pair ID (random) | 34.34 (7.24) | 22.49 | 1 | < 0.001 |
| Individual ID (random) | 33.70 (4.43) | 57.82 | 1 | < 0.001 |
| Sex (ref = female) | −0.37 (1.28) | 0.08 | 1 | 0.777 |
| Age category (ref = 0 d) | | 59273.26 | 5 | < 0.001 |
| 7d | 21.11 (1.31) | 260.17 | 1 | < 0.001 |
| 14 d | 59.33 (1.31) | 2042.51 | 1 | < 0.001 |
| 28 d | 135.53 (1.31) | 10657.89 | 1 | < 0.001 |
| 42 d | 211.47 (1.31) | 25932.63 | 1 | < 0.001 |
| ASM | 246.46 (1.33) | 34403.07 | 1 | < 0.001 |
| Sex × Age category | | 1533.85 | 5 | < 0.001 |
| male × 7 d | −0.54 (1.55) | 0.12 | 1 | 0.729 |
| male × 14 d | −1.26 (1.56) | 0.66 | 1 | 0.417 |
| male × 28 d | −6.24 (1.56) | 17.04 | 1 | < 0.001 |
| male × 42 d | −20.17 (1.56) | 167.68 | 1 | < 0.001 |
| male × ASM | −49.74 (1.57) | 1007.42 | 1 | < 0.001 |
| Treatment (ref = 37.7°C) | −0.26 (1.26) | 0.04 | 1 | 0.841 |
| Treatment × Age category | | 6.56 | 5 | 0.255 |
| 36.0°C × 7 d | −0.51 (1.55) | 0.11 | 1 | 0.740 |
| 36.0°C × 14 d | −0.24 (1.56) | 0.02 | 1 | 0.888 |
| 36.0°C × 28 d | −0.76 (1.56) | 0.24 | 1 | 0.624 |
| 36.0°C × 42 d | −2.28 (1.56) | 2.14 | 1 | 0.144 |
| 36.0°C × ASM | −3.18 (1.57) | 4.12 | 1 | 0.042 |
| $n$ = 2270 measurements of 387 individuals of 91 pairs | | | | |

*ID = identity, ref = reference category, ASM = age at sexual maturity.*

**TABLE 2 |** Summary of (generalized) linear mixed models testing for effects of incubation treatment on sex-specific survival to sexual maturity, and age at sexual maturity, in Japanese quail chicks.

| **Dependent variable: survival to sexual maturity** | | | | |
|---|---|---|---|---|
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 3.23 (0.52) | | | |
| Pair ID (random) | 0.65 (1.18) | 0.30 | 1 | 0.581 |
| Sex (ref = female) | 1.31 (1.12) | 1.37 | 1 | 0.242 |
| Treatment (ref = 37.7°C) | −0.24 (0.73) | 0.11 | 1 | 0.741 |
| Treatment × Sex | −0.84 (1.36) | 0.38 | 1 | 0.537 |
| *n* = 387 individuals of 91 pairs | | | | |
| **Dependent variable: age at sexual maturity (d)** | | | | |
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 53.37 (0.65) | | | |
| Pair ID (random) | 7.23 (2.29) | 9.94 | 1 | 0.002 |
| Sex (ref = female) | −6.50 (0.86) | 57.69 | 1 | < 0.001 |
| Treatment (ref = 37.7 °C) | 0.50 (0.88) | 0.32 | 1 | 0.570 |
| Treatment × Sex | −0.18 (1.26) | 0.02 | 1 | 0.888 |
| *n* = 369 individuals of 89 pairs | | | | |

*ID = identity, ref = reference category.*

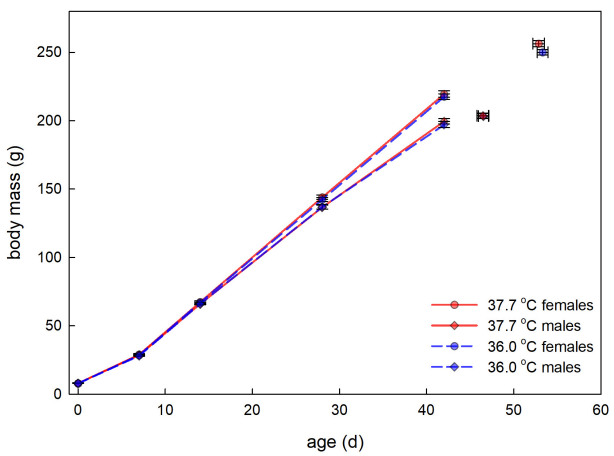


**FIGURE 1 |** Post-hatching body mass (average ± SE) plotted against age for male and female Japanese quail incubated at 37.7°C (*n* = 203 hatchlings) or 36.0°C (*n* = 184 hatchlings). The lines represent the growth in the first six weeks after hatching. Although growth between 7 and 42 days old may appear linear, effects of the treatment were analyzed with age as a categorical variable (see **Table 1**). The final data points (with bi-directional error bars) represent the average age (± SE) and average mass (± SE) at sexual maturity, for each category.

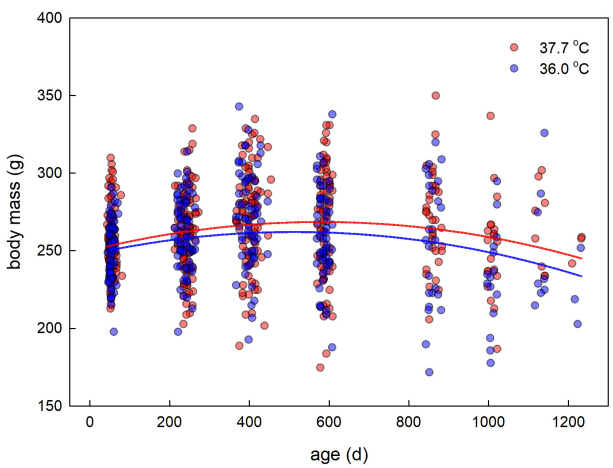


**FIGURE 2 |** Adult body mass plotted against age for female Japanese quail incubated at 37.7°C (*n* = 99 females) or 36.0°C (*n* = 79 females). The lines represent the model prediction (see **Table 3**) for each incubation temperature.

## DISCUSSION

Our unique life-long assessment on the performance of Japanese quail exposed to experimentally manipulated conditions during embryonic development did not provide evidence for effects on performance beyond the period that was manipulated. The lower incubation temperature clearly slowed embryonic development, and reduced the viability of embryos from the first week of development to hatching. Despite the latter effect being just short of statistical significance, both results are in line with general findings in birds (Decuypere and Michels, 1992; DuRant et al., 2013). Since we were specifically interested in long-term effects of our incubation treatment, we did not aim to severely depress hatching success with an even lower incubation temperature. The general pattern of slower and less successful embryonic development with lower incubation temperature can be physically explained by lower temperature providing less energy to fuel biochemical reactions (Gillooly et al., 2002). As such, a lower incubation temperature will represent a resource limitation for the developing embryo, and can be conceptually compared to other types of stressful conditions experienced during development (e.g., decreased food availability, or increased requirement for immunocompetence) in the context of life-history theory.

**TABLE 3 |** Summary of a linear mixed model testing for effects of incubation treatment on age-specific adult body mass in Japanese quail females.

| **Dependent variable: adult body mass (g)** | | | | |
|---|---|---|---|---|
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 251.11 (4.60) | | | |
| Pair ID (random) | 285.54 (65.49) | 19.01 | 1 | < 0.001 |
| Individual ID (random) | 121.01 (31.94) | 14.36 | 1 | < 0.001 |
| Treatment (ref = 37.7°C) | −2.47 (3.37) | 0.54 | 1 | 0.464 |
| Age (d) | 0.06 (0.01) | 59.23 | 1 | < 0.001 |
| $Age^2$ | $-0.55{\cdot}10^{-4}$ ($0.08{\cdot}10^{-4}$) | 53.93 | 1 | < 0.001 |
| Treatment × Age | $-0.73{\cdot}10^{-2}$ ($0.53{\cdot}10^{-2}$) | 1.93 | 1 | 0.164 |
| Age at last sampling (d) | $-0.66{\cdot}10^{-3}$ ($5.47{\cdot}10^{-3}$) | 0.01 | 1 | 0.904 |
| *n* = 740 measurements of 178 individuals of 84 pairs | | | | |

*ID = identity, ref = reference category.*

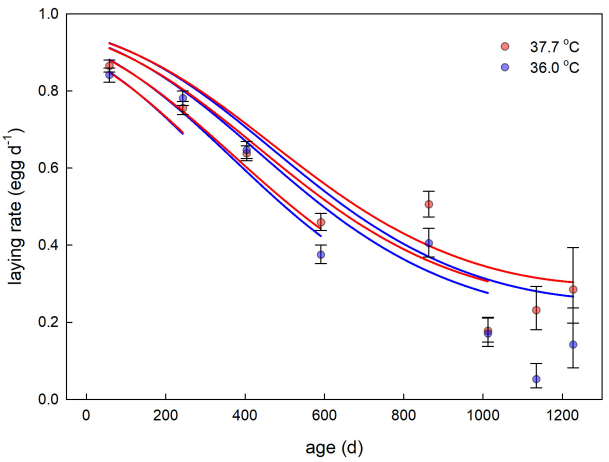


**FIGURE 3 |** Egg laying rate plotted against age for female Japanese quail incubated at 37.7°C (*n* = 99 females) or 36.0°C (*n* = 80 females). Data points and error bars represent averages (± SE) based on the raw data. The lines represent the model predictions (see **Table 4**) for each incubation temperature, and several ages of last sampling, to illustrate the selective mortality of poor layers.

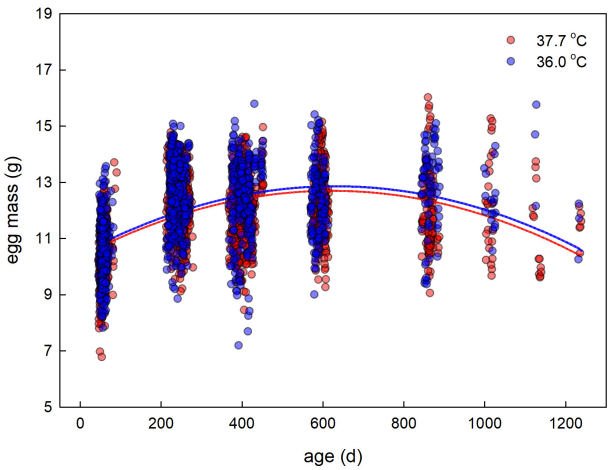


**FIGURE 4 |** Mass of individual eggs laid plotted against age for female Japanese quail incubated at 37.7°C (*n* = 97 females) or 36.0°C (*n* = 79 females). The lines represent the model prediction (see **Table 5**) for each incubation temperature.

As life-history theory is contingent on the existence of resource-based trade-offs between investment in growth, reproduction and (long-term) self-maintenance (Williams, 1966; Kirkwood, 1977; Stearns, 1992), it predicts that a reduced availability of resources to invest in these traits should reduce performance in one or more of these life-history traits. From an evolutionary perspective, it could be hypothesized that late-life performance should get compromised the most by resource limitation during development, because the strength of selection declines with age (Medawar, 1952; Hamilton, 1966). In other words, reduced performance at old age would cause a lesser reduction in fitness than reduced performance at young age. The ability to defer the cost of resource limitation in early life to later in life should thus be favored by natural selection. While we did find a reduced performance during embryonic development, in contrast with expectation, this effect did not carry through, or exacerbate, to post-hatching growth, adult body mass, reproduction and survival.

The absence of effects beyond the period that was manipulated may be explained by the physiological traits that underlie post-hatching performance in growth, survival and reproduction not being sensitive to thermal stress during embryonic development, limiting deferred fitness consequences. Alternatively, the relatively benign conditions under which our study animals lived (e.g., *ad libitum* food, no predation pressure) may have provided them with the opportunity to repair any damage incurred during embryonic development (Omholt and Kirkwood, 2021). Research on livestock selected on extreme productivity, as also relevant to Japanese quail, is equivocal in whether productivity is limited by resource acquisition, or by resource-based trade-offs with somatic maintenance (Douhard et al., 2021). The possibility to repair damage may occur only under favorable environmental conditions, which could be less frequent in nature and explain why long-term adverse effect are frequently reported among

**TABLE 4 |** Summary of a generalized linear mixed model testing for effects of incubation treatment on age-specific laying rate in Japanese quail females.

| **Dependent variable: laying rate (egg $d^{-1}$)** | | | | |
|---|---|---|---|---|
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 1.85 (0.27) | | | |
| Pair ID (random) | 0.23 (0.14) | 2.77 | 1 | 0.096 |
| Individual ID (random) | 0.95 (0.16) | 34.36 | 1 | < 0.001 |
| Treatment (ref = 37.7°C) | −0.02 (0.21) | 0.01 | 1 | 0.920 |
| Age (d) | $-0.55{\cdot}10^{-2}$ ($0.05{\cdot}10^{-2}$) | 149.49 | 1 | < 0.001 |
| $Age^2$ | $-0.21{\cdot}10^{-5}$ ($0.04{\cdot}10^{-5}$) | 29.97 | 1 | < 0.001 |
| Treatment × Age | $-0.17{\cdot}10^{-3}$ ($0.27{\cdot}10^{-3}$) | 0.37 | 1 | 0.544 |
| Age at last sampling (d) | $-0.78{\cdot}10^{-3}$ ($0.33{\cdot}10^{-3}$) | 5.69 | 1 | 0.017 |
| *n* = 4822 days of 179 individuals of 84 pairs | | | | |

*ID = identity, ref = reference category.*

**TABLE 5 |** Summary of a linear mixed model testing for effects of incubation treatment on age-specific egg mass in Japanese quail females.

| **Dependent variable: egg mass (g)** | | | | |
|---|---|---|---|---|
| ***Model*** | ***coefficient (SE)*** | ***χ2*** | ***Δdf*** | ***P*** |
| Intercept | 10.13 (0.18) | | | |
| Pair ID (random) | 0.55 (0.12) | 20.79 | 1 | < 0.001 |
| Individual ID (random) | 0.32 (0.05) | 36.00 | 1 | < 0.001 |
| Treatment (ref = 37.7°C) | 0.10 (0.12) | 0.65 | 1 | 0.419 |
| Age (d) | $0.76{\cdot}10^{-2}$ ($0.02{\cdot}10^{-2}$) | 1523.10 | 1 | < 0.001 |
| $Age^2$ | $-0.61{\cdot}10^{-5}$ ($0.02{\cdot}10^{-5}$) | 1008.05 | 1 | < 0.001 |
| Treatment x Age | $-0.10{\cdot}10^{-3}$ ($0.13{\cdot}10^{-3}$) | 0.61 | 1 | 0.436 |
| Age at last sampling (d) | $0.18{\cdot}10^{-3}$ ($0.22{\cdot}10^{-3}$) | 0.67 | 1 | 0.414 |
| *n* = 2968 eggs of 176 individuals of 83 pairs | | | | |

*ID = identity, ref = reference category.*

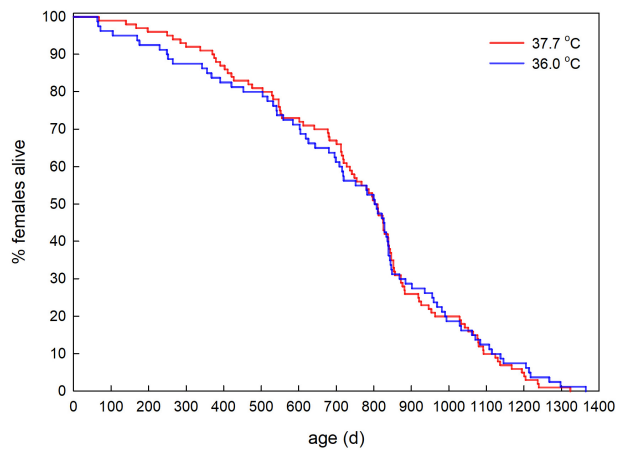


**FIGURE 5 |** Age-specific survival of adult female Japanese quail incubated at 37.7°C (*n* = 100) or 36.0°C (*n* = 80).

animals living in the wild (e.g., Kruuk et al., 1999; Reid et al., 2003; van de Pol et al., 2006; Nussey et al., 2007; Hamel et al., 2009; Spagopoulou et al., 2020). In the wild the acquisition of resources for somatic repair may be more constrained, either directly due to food limitation, or due to a trade-off between foraging and predation risk, causing further trade-offs between repair and late-life performance (Omholt and Kirkwood, 2021). Yet, studies in the wild can often not determine the environmental trait of interest *a priori* and establish causality experimentally. Moreover, no apparent long-term effects of poor early-life environment have been reported also in the wild (Wilkin and Sheldon, 2009; Vedder and Bouwhuis, 2018).

In birds, effects of incubation temperature on post-hatching phenotype are frequently reported, both in captivity and the wild (DuRant et al., 2010, 2012; Hopkins et al., 2011; Nord and Nilsson, 2011, 2021; Ben-Ezra and Burness, 2017; Vedder et al., 2018; Stier et al., 2020). However, studies that tested whether such effects translated to fitness consequences are rare (Hepp and Kennamer, 2012; Berntsen and Bech, 2016). Our study is unique in estimating all components of fitness (i.e., survival to reproduction, age at onset of reproduction, age-specific reproductive performance, and adult survival), without an 'invisible fraction' (Grafen, 1988). Since we did not find an effect of incubation temperature on fitness components beyond hatching success, we caution against over-interpreting the evolutionary relevance of effects on aspects of the phenotype without a clear link to fitness. In addition, we recommend studies that artificially incubate eggs in a conservation context to aim for incubation temperatures that maximize hatching success, as we did not find evidence for antagonistic effects on late-life fitness that could overrule the immediate benefit of surviving the embryonic stage. Nevertheless, we cannot rule

out that fitness costs are transferred to the next generation, for instance if the offspring of the mothers that received the low incubation temperature would show impaired survival (Lemaître and Gaillard, 2017).

We did find that individuals that lived longer had a higher age-specific reproductive rate. A positive correlation between reproduction and survival is also frequently found in the wild, and indicative of heterogeneity in individual quality (Wilson and Nussey, 2010; Vedder and Bouwhuis, 2018). It has been hypothesized that such individual heterogeneity may stem from the quality of the early-life environment positively affecting both reproduction and survival (Vedder and Bouwhuis, 2018). However, our current study shows that heterogeneity in quality is not restricted to the wild. Furthermore, because post-hatching conditions were kept equal between individuals, and the experimentally manipulated incubation temperature had no effect on adult performance, we show that environmental variation may not be required to cause heterogeneity in individual quality. Other sources of individual variation (e.g., genetic or pre-natal maternal effects) may therefore be more promising candidates for future research aiming to explain heterogeneity in quality. For example, inbreeding has strong negative effects on various components of early-life fitness in Japanese quail (Sittmann et al., 1966) and our future research aims to include late-life performance, and estimate the effects of inbreeding over the complete life course. Although, the current experiment did not involve variation in parental age, the scope for transgenerational effects of aging and stress is increasingly recognized (Monaghan and Metcalfe, 2019) and also poses a promising research avenue into understanding the causes of heterogeneity in individual quality.

In conclusion, despite a very comprehensive study to the effects of experimentally manipulated embryonic conditions on performance in growth, survival, and reproduction over the complete life course of a precocial bird, we could not find evidence for effects that occurred beyond the period that was manipulated. We therefore suggest that long-term fitness consequences of the quality of the early-life environment may be less universal than often acclaimed, and caution against translating short-term phenotypic effects to fitness consequences, without knowledge on age-specific reproduction and survival.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

All procedures complied with current German law and did not require ethical review and approval.

## AUTHOR CONTRIBUTIONS

OV and BT: conceptualization and experimental design. OV: data collection. OV and CB: data analysis. OV: writing with feedback from BT and CB. All authors contributed to the article and approved the submitted version.

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# Cancer Susceptibility as a Cost of Reproduction and Contributor to Life History Evolution

*Antoine M. Dujon[1,2†], Justine Boutry[1†], Sophie Tissot[1], Jean-François Lemaître[3], Amy M. Boddy[4], Anne-Lise Gérard[1,2], Alexandra Alvergne[5], Audrey Arnal[1], Orsolya Vincze[6,7], Delphine Nicolas[8], Mathieu Giraudeau[9], Marina Telonis-Scott[2], Aaron Schultz[2], Pascal Pujol[1], Peter A. Biro[2], Christa Beckmann[2,10,11], Rodrigo Hamede[12], Benjamin Roche[1,13], Beata Ujvari[2] and Frédéric Thomas[1]**

[1] Unité Mixte de Recherches, CREEC/CANECEV (CREES), MIVEGEC, IRD 224–CNRS 5290–Université de Montpellier, Montpellier, France, [2] Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, VIC, Australia, [3] CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, Université de Lyon, Université Lyon 1, Villeurbanne, France, [4] Department of Anthropology, University of California, Santa Barbara, Santa Barbara, CA, United States, [5] Institut des Sciences de l'Evolution de Montpellier, Université de Montpellier, Montpellier, France, [6] Institute of Aquatic Ecology, Centre for Ecological Research, Debrecen, Hungary, [7] Evolutionary Ecology Group, Hungarian Department of Biology and Ecology, Babes-Bolyai University, Cluj-Napoca, Romania, [8] Tour du Valat, Institut de Recherche Pour la Conservation des Zones Humides Méditerranéennes, Arles, France, [9] LIENSs,UMR 7266 CNRS-La Rochelle Université, La Rochelle, France, [10] School of Science, Western Sydney University, Penrith, NSW, Australia, [11] Hawkesbury Institute for the Environment, Western Sydney University, Penrith, NSW, Australia, [12] School of Natural Sciences, University of Tasmania, Hobart, TAS, Australia, [13] Fauna Silvestre y Animales de Laboratorio, Departamento de Etología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Mexico City, Mexico*

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***Correspondence:**
*Frédéric Thomas*
*frederic.thomas2@ird.fr*

*† These authors have contributed equally to this work*

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Reproduction is one of the most energetically demanding life-history stages. As a result, breeding individuals often experience trade-offs, where energy is diverted away from maintenance (cell repair, immune function) toward reproduction. While it is increasingly acknowledged that oncogenic processes are omnipresent, evolving and opportunistic entities in the bodies of metazoans, the associations among reproductive activities, energy expenditure, and the dynamics of malignant cells have rarely been studied. Here, we review the diverse ways in which age-specific reproductive performance (e.g., reproductive aging patterns) and cancer risks throughout the life course may be linked via trade-offs or other mechanisms, as well as discuss situations where trade-offs may not exist. We argue that the interactions between host–oncogenic processes should play a significant role in life-history theory, and suggest some avenues for future research.

**Keywords: disease, sexual selection, reproduction, reproductive aging, neoplasia, transmissible cancer, senescence**

## INTRODUCTION

Natural selection shapes organisms to maximize reproductive fitness, and this phenomenon leads to pivotal trade-offs around which all life-history traits ultimately evolve (Williams, 1966; Rose and Mueller, 1993; Roff, 2001; Stearns, 2006; Harshman and Zera, 2007). Exploring the consequences of reproductive trade-offs on organisms has therefore been—and remains—a central topic in

evolutionary biology (Schaffer, 1974; Bell, 1980; Gustafsson et al., 1995; Hamel et al., 2010; Schwenke et al., 2016). There are numerous examples showing that the reproductive allocation of a given event correlates negatively with both short-term survival and/or future reproductive performance (Stearns, 2006; Hamel et al., 2010). Similarly, reproductive costs can be paid in the long run, through an earlier and or steeper actuarial and reproductive aging (Lemaître et al., 2015). Trade-offs between reproductive effort and subsequent survival can occur for a variety of reasons, one of them being that reproductive activities are energetically demanding, thus the amount of energy an individual allocates toward reproduction reduces its allocation to health maintenance (Kirkwood, 1977; Maklakov and Immler, 2016). For instance, reproductive activities and allocation to immune function are often mutually constraining: increased reproductive activity may limit immune performance, which in turn can enhance vulnerabilities to infections (Sandland and Minchella, 2003; French et al., 2007; Knowles et al., 2009; Fedorka, 2014; Schwenke et al., 2016). Allocation of resources away from soma to reproduction might also result in a decreased capacity to cope with damage caused by stress and toxicity, and/or be associated with detrimental by-products of metabolism, e.g., the production of damaging reactive oxygen species (Metcalfe and Monaghan, 2013, but see Blagosklonny, 2010). Finally, some of the genes selected for fitness conferring advantages during early life (e.g., increased reproductive output) may also have other functions; for example, carrying alleles that support reproduction may at the same time increase the risk of pathologies later in life (i.e., antagonistic pleiotropy) (Williams, 1957; Leroi et al., 2005; Madimenos, 2015; Austad and Hoffman, 2018; Gunten et al., 2018).

Apart from being a leading cause of human death worldwide, cancer is a biological process that appeared with the evolution of metazoans during the late Precambrian (Domazet-Lošo and Tautz, 2010; Nunney, 2013). Cancer occurs when individual cells become malignant, i.e., lose their normal cooperative behavior, become insensitive to host controls, proliferate in an uncontrolled fashion, and spread from primary tumors to surrounding tissues and then to distant organs (i.e., metastasis), thereby causing morbidity and potentially death (Hanahan and Weinberg, 2011). Chronological age is indisputably the most significant risk factor (in terms of incidence) for developing metastatic cancer, but it is also well established that oncogenic processes frequently exist at sub-clinical levels earlier in life (in humans and other animals, Folkman and Kalluri, 2004; Bissell and Hines, 2011; Madsen et al., 2017), and in fact represent a long continuum between precancerous lesions to invasive forms (Maley et al., 2017). Recent advances in oncology suggest that pathogens, parasites, viruses, or transposable elements may be the most common causes of cancer in wildlife, while second-hand smoke, nutritional challenges, breeding stress, UV radiation, and chemicals in the environment causing somatic mutations may be most important for pets and humans (Aktipis and Nesse, 2013; Giraudeau et al., 2018; Pesavento et al., 2018). Since cancer is observed in almost all branches of multicellular life, from *Hydra* to whales (Leroi et al., 2003; Aktipis et al., 2015; but see Azpurua and Seluanov, 2013; Fortunato et al., 2021), we hypothesize that cancer should be a major mediator of life-history trade-offs and a contributor to life-history evolution.

The short- and long-term fitness consequences of oncogenic manifestations early in life, at a life-history stage where individuals are expected to maximize their allocation of resources toward growth and reproduction, remain poorly documented (Vittecoq et al., 2013; Thomas et al., 2018). In addition, the precise reasons why oncogenic processes—depending on organs, individuals, and/or species— evolve at variable rates inside the body are not yet well understood, although there is clear evidence that a variety of internal and external parameters are important (e.g., sex, age, immune status, organ ecology and microenvironment, energetic capacity, stress, environment, Tevfik Dorak and Karpuzoglu, 2012; Henry et al., 2015; Thomas et al., 2016; Hochberg and Noble, 2017; DeGregori et al., 2018; Biro et al., 2020; Vibishan and Watve, 2020). Evolutionary ecologists have, until recently, largely neglected the importance of cancer cells for animal ecology (Thomas et al., 2017). There are more than 100 types of cancer (grouped into five major categories: carcinoma, sarcoma, myeloma, leukemia, and lymphoma) that can affect almost every part of the body (Weinberg, 2007). In addition to malignant tumors, multicellular organisms often harbor benign neoplasms (Boutry et al., 2022b). Benign neoplasms have received less attention than malignant tumors because they have less often obvious and serious impacts on the patient/host health, even if noticeable exceptions exist (see Boutry et al., 2022b for a recent synthesis).

Tumoral processes, especially malignant ones, can be detrimental to host fitness by imposing direct costs on it. For instance, malignancies affecting reproductive organs, like cervix, uterus, endometrial, ovary or testicular cancers, can severely compromise their host's reproductive potential by triggering reproductive aging or inducing infertility (Brinton et al., 2004, 2005; Paduch, 2006; Singh et al., 2011; Hanson et al., 2017). Another well-known direct cost of (notably metastatic) cancers on host fitness is due to their detrimental effect on survival. While the survival cost of cancer is largely documented for human and domestic animals, less information is available for wildlife species. Recent studies from zoo animals showed that cancerous processes are however widespread (see Vincze et al., 2022). The lack of information in the wild is likely due to the fact that tumor-bearing individuals, often in poorer condition than healthy ones, have increased probability to die early, being victims of predation or infections (Boutry et al., 2022a). Thus, cancer is indirectly costly when tumor-bearing individuals are considered within their ecosystems. Although experimental evidence would be welcome, it is also likely that tumor-bearing individuals should be less attractive for sexual partners, and/or will have lower ability to deliver good parental care (Vittecoq et al., 2015).

As with other fitness-reducing factors, evolutionary theory predicts that metazoans are under selective pressure to: (i) avoid the source of malignancies in the first instance, (ii) prevent tumoral progression once initiated, and finally (iii) alleviate the fitness costs if further cancer development is not preventable (Ujvari et al., 2016). These strategies necessarily involve immediate and/or long-term costs that are traded against other functions (Jacqueline et al., 2017a), even if the evolution

of cancer defenses is predicted to be variable between species, depending on their ecology. For instance, tumors are common in laboratory mice, but are rarely observed in wild populations. While this difference between natural and artificial environments may in part be due to differences in environmental exposure to cancer inducing agents, it also results from the fact that the average lifespan of a wild mouse (a prey species) is only a few months. While somatic mutations contributing to cancer in laboratory mice may be common, historically, there may have been little selection on immune processes that prevent, or slow cancer because wild mice rarely live long enough to experience tumor formation (Perret et al., 2020).

Thus, cancerous processes, through their direct costs on hosts and/or most often through the costs of host defenses, have the potential to impose life history tradeoffs (Aktipis et al., 2013; Brown and Aktipis, 2015; Muller, 2017). We discuss here the idea that malignant processes are crucial to consider when studying life-history traits because malignant dynamics—and hence their consequences on health and survival—are likely to be linked in multiple ways with the age-specific reproductive activities of organisms (see **Figure 1**). While there have been many review papers linking host evolutionary ecology to cancer (e.g., Peto's paradox), to our knowledge, our paper is the first to primarily focus on the associations between cancer and reproduction. This synthesis highlights the underestimated role of oncogenic processes in shaping the multiple facets of the age-specific reproductive biology of multicellular hosts.

## GENES PROMOTING BOTH FERTILITY AND CANCER RISK

One mechanism that can link together reproduction with increased susceptibility to cancer is through genes that have direct effects on both. Like several other biological processes detrimental to health, malignant dynamics can also be embedded in the antagonistic pleiotropy theory (e.g., Crespi and Summers, 2006; Carter and Nguyen, 2011; Jacqueline et al., 2017a; Lemaître et al., 2020a). At the genetic level, for instance, some alleles that increase cancer risk as a secondary effect of their positive roles on reproductive success have been identified; a good example is the *Xmrk* melanoma-promoting oncogene in the fish *Xiphophorus cortezi* (**Figure 2**). Despite significant deleterious cancer-induced effects leading to a shorter lifespan, this oncogenic allele persists in natural populations presumably because it is also associated with a larger body size, which increases the individual's reproductive success through mate competition. This oncogene is also directly correlated with aggressive behaviors (Fernandez, 2010). In addition, the presence of melanoma on the male's caudal fin intensifies the spotted caudal melanin pattern, which increases female preference for these mates (Fernandez and Morris, 2008; Summers and Crespi, 2010).

Another example concerns mutations on the tumor-suppressing gene BRCA, which is responsible for DNA repair. While women carrying *BRCA1/2* mutations have higher cancer incidence and higher mortality, one study reported that *BRCA* mutation carriers born in Utah prior to 1930 had naturally higher fertility compared to controls (before the advent of modern contraception) (Smith et al., 2012; see however, Oktay et al., 2015; Daum et al., 2018). The precise mechanisms behind this phenomenon are not completely understood, and perhaps are simply the consequence of genetic bottlenecks. However, they could also involve a trade-off between cancer suppression and tolerance for invasive fetal cells (Aktipis, 2020). Placentation in female eutherian mammals, like cancer metastasis, is indeed an invasive process that involves the transplantation of cells into new environments. Females in these species would therefore be potentially more vulnerable to cancer (Kshitiz et al., 2019). This hypothesis seems to be supported at the interspecific level, since species of mammals with more invasive placentation such as felines and canines are also more vulnerable to malignancies (D'Souza and Wagner, 2014). However, Boddy et al. (2020) did not confirm this tendency, and found instead at the interspecific level a positive relationship exists between litter size and prevalence of malignancy. Beyond these examples, the search for positive selection signals on mutations occurring in cancer suppression genes seems to be promising for the identification of candidate genes responsible for both fertility enhancement and cancer risk (Kang and Michalak, 2015).

The fact that cancer genes are not purged by natural selection can also be driven by antagonistic coevolution processes that occur in cases of conflict between evolutionary agents (Graham, 1992; Crespi and Summers, 2006). Some of these conflicts are related to reproductive activities, e.g., parent–offspring conflict, maternal–fetal conflict, sexual conflict, and/or sexual selection (Haig, 2015). The genes involved in such dynamics generate evolutionary disequilibriums, molecular-level arms races, and tugs-of-war over cellular resources, and they may increase susceptibility to cancer as a pleiotropic by-product of their role(s) in antagonistic coevolution (Crespi and Summers, 2006). Situations of sexual conflict, i.e., when the fitness of one sex comes at the expense of the other sex, can occur when the genes involved in rapid cell proliferations—which allow rapid growth or wound healing—are due to sexual selection being more advantageous for fighting males than for females. The optimum trade-off between DNA repair and cell proliferation rate, which also relies on the *BRCA1* and *BRCA2* alleles (Yoshida and Miki, 2004), is therefore likely to vary between males and females, generating sexually antagonistic selection. Oncogenic consequences for females, such as breast and/or ovarian cancers, could result from a disruption of the trade-off via a BRCA gene mutation in a somatic cell, leading to proliferation with less-effective DNA repair. Total cancer risks arising from perturbations to maternally and paternally opposed growth regulation have yet to be determined (Frank and Crespi, 2011), but exploring this hypothesis with comparative analyses seems promising. In particular, considering species that vary in the intensity of male sexual selection relying on body size and aggressive fights could be of interest since the differential optimum values for the repair–proliferation trade-off between sexes are expected to be somewhat different.

It is also thought that genes contributing to gamete production (rate and/or quality) may use pathways that are highly beneficial for cancer cells able to co-opt or subvert them during somatic evolution. These pathways could allow rapid cell proliferation

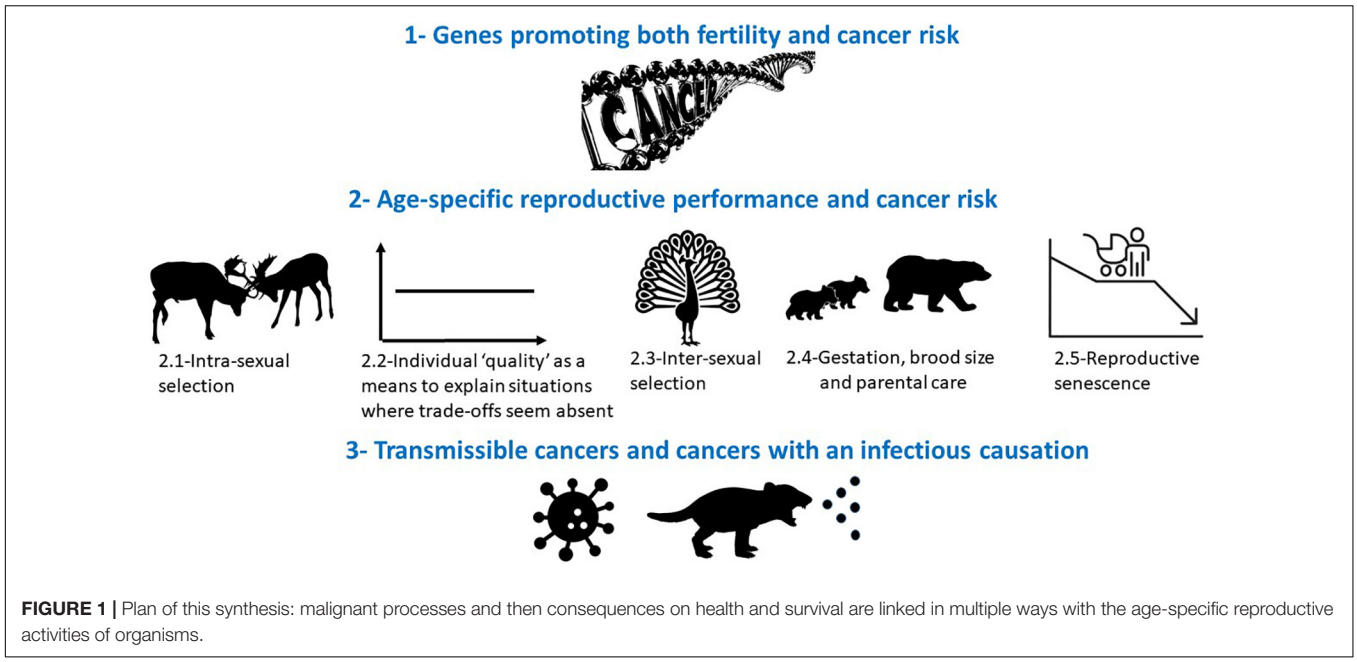


**FIGURE 1 |** Plan of this synthesis: malignant processes and then consequences on health and survival are linked in multiple ways with the age-specific reproductive activities of organisms.

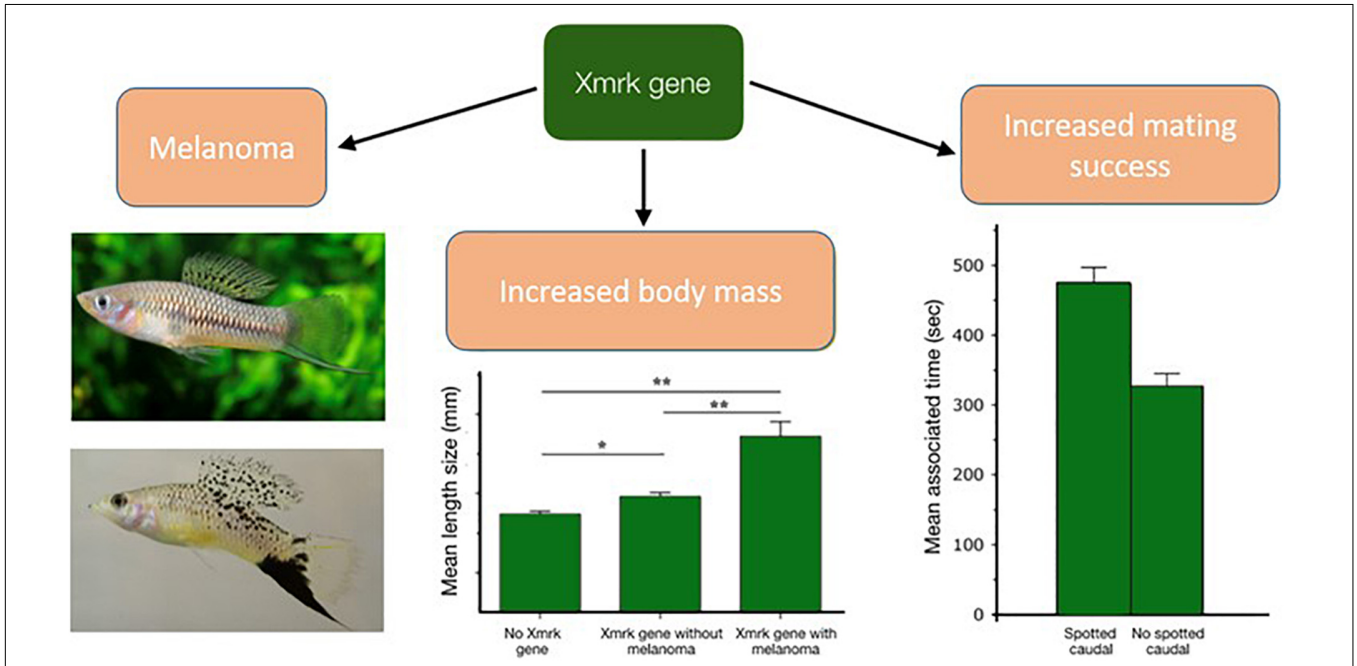


**FIGURE 2 |** Antagonistic pleiotropy and oncogenic congenital mutations: the *Xmrk* melanoma-promoting oncogene in the fish *Xiphophorus cortezi.* Sexual selection processes may sometimes favor cancer promoting oncogene alleles, as this is observed in the fish *Xiphophorus* developing melanoma (bottom Photo). Despite shorter lifespan due to the deleterious cancer-induced effects, the oncogenic *Xmrk* allele is not eliminated from natural populations of *Xiphophorus*, because several associated benefits early in life outweigh late costs (i.e., antagonistic pleiotropy). *Xmrk* allele is indeed associated to a larger body size and higher aggression, which increases individual's reproductive success through mate choice and competition for mates. The source and credit for the fish photos: Johnny Jensen (up) and Andre Fernandez (down). Figure modified from Fernandez and Morris (2008). $^{*}P < 0.05$, $^{**}P < 0.01$.

and/or avoidance of control by tumor suppressors or the immune system (Crespi and Summers, 2006). For example, *SPANX* genes in primates, which show evidence of strong positive selection, are involved both in spermatogenesis and in melanoma progression by promoting cancer cell growth (Kleene, 2005). More recent studies also support the hypothesis that some genetic pathways of spermatogenesis, whose evolution is governed by responses to sexual selection and intra-sexual conflict, are the same as those used by cancer cells to increase their survival and replication (genes such as *BRIP1*, *BUB1B*, *KTN1*, and *RANBP2*)

(Vicens and Posada, 2018). It has also been suggested that the CAG repeat region of the androgen receptor could be a locus of antagonistic pleiotropy in the context of sexual selection and sexual conflict (Summers and Crespi, 2008). Short repeats are associated with increased fertility at the phenotypic level, but there is also evidence that somatic evolution of malignant cell lineages during cancer progression are often associated with a repeated pattern of shortening of the CAG repeat, *a priori* because of positive selection among cell lineages.

## AGE-SPECIFIC REPRODUCTIVE PERFORMANCE AND CANCER RISK

### Intra-Sexual Selection

As mentioned before, antagonistic pleiotropy is when one gene has opposite effects on fitness at different ages, such that their effects are beneficial in early life, when natural selection is strong, but harmful at later ages, when selection weakens. In a theoretical study, Boddy et al. (2015) investigated the possible links between cancer risks and the level of intraspecific competition, assuming that an underlying resource-based trade-off between competitiveness and allocation into cancer defenses exists. As has been empirically observed (see Clocchiatti et al., 2016 for an example in humans), the model first showed that cancer prevalence is expected to be lower in females than in males, since mating competition usually has greater reproductive payoffs for males than for females (Clutton-Brock and Vincent, 1991) and also because of the immune-enhancing effect of estrogen (Taneja, 2018; even if the picture is more variable in humans, Mulder and Ross, 2019). Although experimental evidence is currently lacking, the higher male susceptibility is likely to be exacerbated when they grow faster and/or have to develop and maintain extravagant secondary sexual traits. This should theoretically be the case because the required higher cell proliferation itself increases cancer risk (especially in body areas with the greatest cellular divisions, e.g., testes cancer, antleromas). For instance, the insulin/insulin-like growth factor (IGF) signaling pathway to mTOR is essential for the survival and growth of normal cells but also contributes to the genesis and progression of cancer, increasing cell proliferation and anti-apoptosis processes (LeRoith and Roberts, 2003; Floyd et al., 2007). Another reason is because of a diversion of resources from somatic maintenance (e.g., DNA repair or immune defenses). A potential self-maintenance mechanism suffering shortfall could be the antioxidant machinery, when strenuous activity (e.g., mating competition, reproductive effort, maintenance of costly sexual signals) leads to an increased production of reactive oxygen species causing oxidative stress. Even if empirical data have not been always consistent, oxidative stress has been viewed as an important physiological costs of reproduction (Blount et al., 2016; Pap et al., 2018) and it is well known to participate in cancer development by promoting cancer initiation and progression through induced DNA damage, leading to mutagenesis, and by affecting DNA methylation patterns or the expression of oncogenes (Van Remmen et al., 2004; Kreuz and Fischle, 2016). Moreover, as recently highlighted by Ujvari et al. (in press), telomere dynamics could also occupy a pivotal position in these processes. Indeed, allocation toward the maintenance of costly sexual signals often alters oxidative metabolism (Hill, 2014), which can, in turn, provoke telomere erosion (Monaghan and Ozanne, 2018) and influence the emergence of oncogenic mutations. It is predicted that natural selection should favor the expression of secondary sexual traits over the cost of telomere dysfunction until the organism reaches its maximum reproductive potential, even if this is likely to promote malignant progressions beyond this (Taff and Freeman-Gallant, 2017; Pepke and Eisenberg, 2021). In a recent study, Wang et al. (2019) studied the genetic basis of antler regeneration in ruminants; antlers are among the fastest-growing bone in the animal kingdom. In red deer (*Cervus elaphus*), for instance, antlers regrow annually and reach up to 30 kg, with growth rates of ∼1.7 cm/day, which necessitates a rate of cell proliferation that surpasses classical cancerous tissue growth. Wang et al. (2019) found that the genes underlying the expression of these large sexual ornaments are among those that both promote and suppress cancer (**Figure 3**). Specifically, there was a higher correlation between the gene expression profiles of antlers and bone cancer such as osteosarcoma than between those of antlers and normal bone tissues, suggesting that antler growth and oncogenesis rely on similar developmental programs. Cancer is typically a pathology arising from perturbations to a precarious balance between strongly opposed growth promoters and growth repressors (Aktipis, 2020). However, these authors discovered that contrary to the situation in osteosarcoma, where neoplasms progress unchecked, antler growth was tightly regulated by the activity of cancer-controlling genes (both tumor-suppressing and tumor-growth-inhibiting genes), suggesting that antler growth is mechanistically equivalent to a controlled form of bone cancer growth. Cervids generally display a very low risk of cancer (Vincze et al., 2022). Ideally, the same genes promoting cancer prevention (tumor-suppressors like BRCA, TP53, BUB1, BUBR1, TGF-βRII, Axin, DPC4, p300, and PPARγ which generally play a role in controlling the cell cycle or DNA repair) would be selected for and likely conserved. However, they would share the same pathway used by cancerous cells because the same genes are used in cell division. Given that antlers are lost every year, we might speculate that this could potentially be a defense mechanism. In line with this hypothesis, a recent comparative analyses of cancer risk across almost 200 species of mammals highlighted that Artiodacyla such as Cervids are much less prone to cancer than other mammalian orders (Vincze et al., 2022).

The apparent lack of a correlation between sexual ornament size and cancer rate may be explained by mechanisms that offset the increased cancer risk. As possible supportive evidence, albeit in a different ecological context, Thomas et al. (2020) argued that strong ongoing artificial selection in domestic animals has sometimes resulted in extreme phenotypic responses favoring a higher incidence of cancer. However, there is also evidence for effective anti-cancer defenses in several domesticated animals, suggesting that artificial selection may also favor the evolution of compensatory anticancer defenses (see also Ibrahim-Hashim et al., 2020). More research in wild species, especially at the intraspecific level, would be necessary to evaluate how robust and

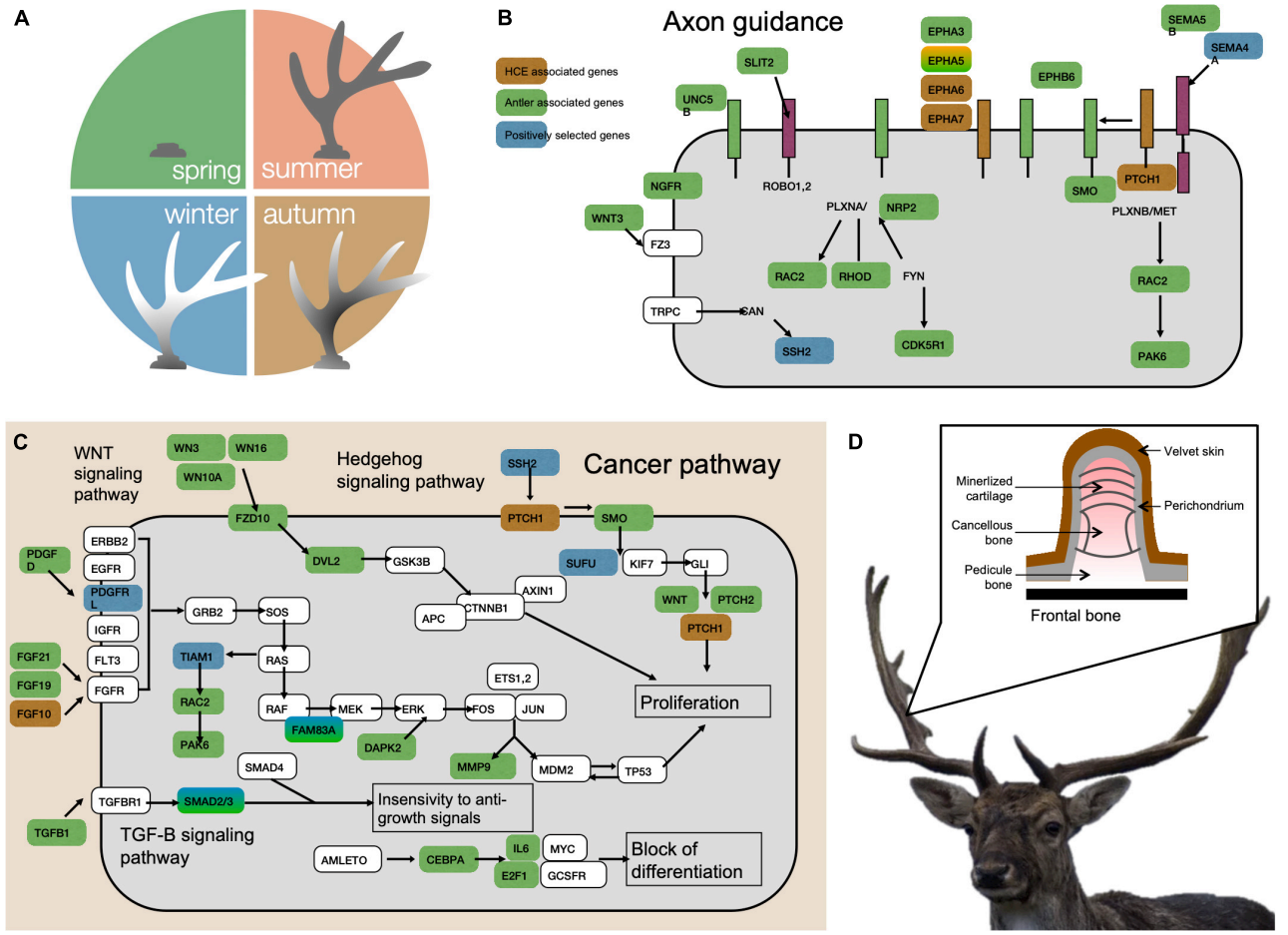


**FIGURE 3 |** Antler regeneration, neurogenesis and oncogenesis pathways. **(A)** Annual antler regeneration cycle in red deer. Antlers, after shed in winter regenerate in spring, and then grow most rapidly in summer. In autumn, they calcify and shed their velvet. For **(B)** (Axon guidance pathways) and **(C)** (Oncongenesis pathways): blue, genes positively selected in cervids; yellow, cervid-specific HCE associated genes; green, antler-specific expression genes. **(D)** Antler anatomy. **(C)** The anatomy of the antler. The source and credit for the red deer: Pixabay. Figure modified from Wang et al. (2019).

buffered these compensatory protections are. Indeed, because sexual selection processes may be intense in natural populations depending on the ecological contexts, it could be expected that animals benefiting from abundant resources might develop secondary sexual traits whose expression level is higher than that corresponding to their cancer defenses. A rapid evolution in this direction would then drive genotypes away from the optimum, which would result in an increased risk of cancer, at least until the species or population adapts to the changes (Abegglen et al., 2015; Boutry et al., 2020). Such kind of mismatch scenario could potentially be investigated in habitat when wildlife artificially benefits from excessive quantity of food. In humans, it is well established that good nutrition has promoted growth in recent decades, allowing individuals to grow taller (Grasgruber et al., 2014). However, it is also known that cancer risk increases with adult height, at least in part because the number of cells correlates positively with stature, while cancer defenses apparently remain the same (Nunney, 2018). It is unlikely that mechanisms will evolve to prevent large stature because of the sexual selection benefits associated with height in males (Stulp et al., 2013) as well as the reduced risks of obstetric complications in females (Guégan et al., 2000), and also because most oncogenic consequences will occur relatively late in life. However, such processes, at least in wild species, could result in positive feedback cycles in oncogene evolution, leading to improved tumor suppression, greater developmental precision and complexity, and further adaptive changes driving pleiotropic oncogene evolution (Crespi and Summers, 2006). Domestication of animals by humans may provide some support for the hypothesis that a mismatch between cancer risk and cancer defenses may promote malignant proliferation. For example, the artificial selection for size in dogs results in higher incidences of bone cancer in larger breeds compared to smaller ones (Grabovac et al., 2020) because selection for genes responsible for height (and hence a larger number of cells) was not accompanied by selection for more efficient cancer defenses (Thomas et al., 2020). In a related vein, pediatric cancers in humans most often concern three compartments, brain, blood and bone, that have undergone

rapid phenotypic changes in their developmental trajectories along the human lineage (Leroi et al., 2003). In current times, the risk of mismatch can also be exacerbated because anthropic activities result in higher pollution by mutagenic substances in ecosystems (Giraudeau et al., 2018) that—all things being equal—lead to unprecedented risks of cell derailment rates in organs that undergo intense cell divisions.

Although potentially specific to certain populations (see for instance Trumble et al., 2014), testosterone in males has frequently been associated not only with a greater investment in mating behavior (Wingfield et al., 1990; Peters et al., 2008; Summers and Crespi, 2008; Dixson, 2015), but also (although still debated, Michaud et al., 2015) with a higher risk of prostate cancer (e.g., Alvarado, 2013 for males in human polygynous societies). This correlation potentially supports the hypothesis of a trade-off, where higher testosterone levels would allow males to engage in more short-term mating with different partners but at the cost of a higher long-term risk of prostate cancer. It is however, difficult to separate hormonal effects and higher exposure to sexually transmitted carcinogenic viruses also affecting prostate tissues, a risk that increases with the number of sexual partners (e.g., Moghoofei et al., 2019). Further research is needed to decipher the interactions between sexual hormones, the immune system, and cancer development in wild species. Promising models could be social species, especially those for which there are differential hormone levels and cancer risks between dominant and subordinate individuals (Jacqueline et al., 2017a).

## Individual 'Quality' as a Means to Explain Situations Where Trade-Offs Seem Absent

Although trade-offs between reproduction and the ability to ward off cancer are expected based on long-standing life history theory (citations above), we should not always expect that reproductive costs are evident or detected. For example, investment into large antlers by Artiodactyla (discussed above) may not create detectible energy trade-offs and increased cancer risk if dominant individuals monopolize resources prior to breeding as is often the case in some species. That is, some individuals of high 'quality' possess abundant energetic resources that permit them to allocate energy to competing demands obscuring trade-offs. Similarly, some of the dramatic anti-cancer adaptations found in domestic animals (Thomas et al., 2020) may be also explained by the fact that abundant food permits simultaneous allocation of energy to reproduction and anti-cancer functions, again obscuring trade-offs. These views are consistent with theory showing that when variation in energy acquisition among individuals is greater than variation in allocation within individuals, competing life history traits will no longer be negatively correlated among individuals (van Noordwijk and de Jong, 1986). Indeed, reviews show energy expenditure on activity, reproduction and maintenance metabolism can often be positively correlated at the among individual level for domestic and laboratory reared animals (Biro and Stamps, 2008, 2010).

## Inter-Sexual Selection

In addition to intra-sexual selection, inter-sexual selection through mate choice is central in the theory of sexual selection (Andersson and Simmons, 2006). For many years, it was expected females of many species chose to mate with old rather than young males, because older males often provide better resources (e.g., territories, parental care) and/or pass good genes on to their offspring (Brooks and Kemp, 2001). However, these theoretical predictions are now thoroughly revisited in the light of recent studies demonstrating the under-estimated occurrence of male reproductive aging, as well as the detrimental consequences of this process on female fitness (Lemaître and Gaillard, 2017; Monaghan and Metcalfe, 2019; Monaghan et al., 2020; Segami et al., 2021). Empirical studies suggest that female preference toward young males could be a pervasive mating tactic in the living world (e.g., Verburgt et al., 2011; Vanpé et al., 2019). Here, we propose that mating preferentially with younger males might also be adaptive in terms of a decreased cancer risk in the progeny. One reason is that males accumulate deleterious mutations in their germ-line at an ever-increasing rate as they age (Beck and Promislow, 2007), thereby reducing the quality of genes passed on to the next generation, which potentially favors cancer (e.g., see Choi et al., 2005 for an example that older paternal age increases the risk of breast cancer in female offspring). In addition, in several species – at least in humans and chimpanzees – sperm telomere length is positively correlated with male age, leading to a positive correlation between paternal age at conception and offspring telomere length (Eisenberg and Kuzawa, 2018; Eisenberg, 2019; Eisenberg et al., 2019). Longer telomeres in descendants of old fathers are likely to predispose the novel generation to a higher risk of cancer since cells have a greater chance to accumulate bad mutations before replicative senescence occurs and eliminates them (e.g., Aviv et al., 2017). Theoretical models aiming to predict age-based mating preferences in females based on the benefits-costs balance provided by both young and old partners should now fully consider the risk of cancer in the progeny. For instance, it may remain evolutionarily beneficial for females to produce a progeny whose lifespan will be shortened by cancer in species where old males provide more resources, especially if the cancer-induced death can occur relatively late in the life of offspring (i.e., once most of their reproduction has been achieved). In addition, given the heritability of height (Yang et al., 2010), further studies should also explore the possible oncogenic consequences for tall partner preference in humans.

## Gestation, Brood Size, and Parental Care

In numerous species, reproduction is costly for females in terms of energy, nutrients, and metabolic adjustments, which may lead to faster aging and reduced longevity when reproductive effort increases (Westendorp and Kirkwood, 1998; Lemaître et al., 2015; Jasienska, 2020). Physiological consequences of elevated reproductive expenditure are also observed on biological markers of aging. For instance, in species for which males also have a high allocation of resources toward parental care, e.g., common terns (*Sterna hirundo*), where males are primarily responsible

for chick feeding, a negative association between reproductive success and telomere length is observed only in males (Bauch et al., 2016). The direct and/or indirect consequences of these kinds of reproductive costs on oncogenic process dynamics have not yet been fully explored for most species. Pregnancy in women, at least in Western populations, seems to have a dual effect on breast cancer risk (Hsieh et al., 1994). While full-term pregnancies in early life (<30 years) reduce breast cancer risk in the long-term (Albrektsen et al., 2005; Hurt et al., 2006), in the short term they boost the development of oncogene-activated cells into tumors and/or promote a metastatic cascade (Lambe et al., 2002; Lyons et al., 2011), which transiently increases cancer risk. The most common oncogenic progressions observed during pregnancy are malignant melanoma and lymphomas, leukemia, and breast, ovary, cervix, colon, and thyroid cancers (Pavlidis, 2002; Oduncu et al., 2003). The cancer risk is highest within the first 5 years after giving birth, and the risk of breast cancer in parous women is increased for more than 15–20 years compared with nulliparous women (Nichols et al., 2019). Multiple factors are likely responsible for this higher vulnerability to cancer during pregnancy, such as the strategic modulation of the maternal adaptive immune system during the first trimester and a relatively high inflammatory status (Fessler, 2002; Abrams and Miller, 2011; Lyons et al., 2011; but see Hové et al., 2021). The pathophysiology of cancer associated with pregnancy also includes factors like hormonal changes, permeability, and vascularization (D'Souza and Wagner, 2014; Hepner et al., 2019).

Enhanced reproductive effort through increased parental care has often been linked to concomitant or subsequent reduced immune-competence (Nordling et al., 1998), which in return should promote the proliferation of malignant cells (Jacqueline et al., 2017a). However, it seems likely in nature that reduced immunocompetence would first increase the frequency of infections that are detrimental to survival in the short term, long before malignant processes that escape immune-surveillance could have an effect of survival. The fact that detrimental oncogenic consequences in the wild are hidden because of deaths resulting from infectious processes does not mean that they are not significant, nor can we exclude the possibility that oncogenic consequences may interact with infectious dynamics (e.g., see Goldszmid et al., 2014; Jacqueline et al., 2017b, 2020). The oncogenic consequences resulting from trade-offs with enhanced parental care, if any, will be mainly observed in parasite- and predator-free environments such as zoos, provided breeding activities continue (see also Tanaka et al., 2020).

While high reproductive investments can potentially result in increased cancer risks for individuals due to immediate trade-offs, it is also expected that natural selection can fix these problems on the long term. For instance, Brown and Aktipis (2015) suggested in a theoretical study that in cases of extended parental care, as well as with cooperative breeding systems, selection can favor cancer suppression into old age. At least for the proof of concept, artificial selection in the context of domestication illustrates that enlarging reproductive efforts may select for compensatory adaptations or additional defenses (Thomas et al., 2020). For example, while the ancestor of domesticated hens may live for 20–30 years and produce only small numbers of fertile eggs during a limited period of the year, strong artificial selection leading to the domesticated jungle fowl hen (*Gallus gallus domesticus*) has resulted in animals with a short lifespan and prolific daily ovulation and egg laying. Malignant ovarian cancer in domesticated chickens is frequent, but there is a five-fold variability between strains in the incidence of the disease (Johnson and Giles, 2006), suggesting that anticancer responses may have subsequently evolved in certain strains. Similarly, in dairy cows and goats that have been selectively bred for mammary gland growth and milk production, there is paradoxically a low occurrence of mammary tumors compared to domestic carnivores (Munson and Moresco, 2007), suggesting once again that anticancer mechanisms compensating for increased risks of lobular alveolar growth and hence malignancies have been concomitantly and/or subsequently selected. Further studies would be necessary to explore how relevant these phenomena are in wild species currently experiencing alterations in the reproductive biology because of climate change (e.g., Winkler et al., 2002; Pankhurst and Munday, 2011).

## Reproductive Aging

The evolutionary ecology literature is replete of studies documenting reproductive aging (i.e., the decline in reproductive performance with increasing age, also coined reproductive aging) in females (see Holmes et al., 2003; Lemaître and Gaillard, 2017 for reviews). The decline in litter size from 10 years of age onward observed in Alpine marmot, *Marmota marmota* (Berger et al., 2015) constitutes one of the numerous examples of reproductive aging, and many other of reproductive traits (e.g., litter size, birth rate, juvenile survival) have been show to decrease with age. Nowadays, the common view is that female's reproductive aging is the rule rather than the exception, at least in endotherm vertebrates (more than 60% of the studies species in both birds, Vágási et al., 2021 and mammals, Lemaître et al., 2020b) even if reproductive aging trajectories remain highly variables both within and across species (Reid et al., 2010; Lemaître et al., 2020b). Reproductive aging is obviously particularly pronounced in species displaying menopause (see Péron et al., 2019), a cessation of the reproductive functions that occurs many years before death. In addition, there is now pervasive evidence that reproductive aging is common among males of various species. Indeed, while evidence that male fertilization efficiency decreases with age have been documented for a long time in human (see Johnson et al., 2015) and laboratory rodents (vom Saal et al., 1994), an increasing number of studies are now reporting male reproductive aging in semi-captive or wild populations of animals. Such decline in reproductive performance have been observed using reproductive success metrics (e.g., mating success, Raveh et al., 2010) but also the conspicuousness of secondary sexual traits (e.g., Perrot et al., 2016, or the quality of the ejaculates, Preston et al., 2011). However, while the study of reproductive aging is showing an unprecedent infatuation, the link between both reproductive aging and oncogenic processes are yet to be deciphered.

These two processes, reproductive aging and oncogenic processes, can share similar proximate origins. As emphasized in the sections above, an elevated reproductive expenditure

could be responsible for an increased risk of cancer (Boddy et al., 2015). Interestingly, there is compelling evidence that such substantial allocation to reproduction during early-life is also detrimental in terms of an earlier/stronger reproductive aging (e.g., Nussey et al., 2006 and Lemaître et al., 2014, for examples in females and males red deer, *Cervus elaphus*, respectively) and the physiological pathways that have been suggested to mediate the link between early- and late-life reproductive performance are strikingly similar to the one suggested to be involved in the reproductive effort-cancer trade-offs (e.g., oxidative stress, telomere attrition, see Kalmbach et al., 2015). However, this does not preclude any complex interactions between reproductive aging and oncogenic processes and understanding whether the occurrence of cancer is a cause, a consequence or is – to some extent – independent to the aging process remains a long-standing question (de Maghalaes, 2013; Thomas et al., 2018). Moreover, it is noteworthy that the cancer-reproductive aging dynamic is likely to be different between males and females for which the age-specific cancer rate of reproductive organs show striking differences (see de Maghalaes, 2013).

The various risk of cancers associated to the physiological pathways modulating the reproductive sequence (e.g., pregnancy in mammals) might have constituted a selected pressure on the evolution of reproductive aging patterns. For instance, it has recently been proposed (Thomas et al., 2019a) that menopause in humans (and in a few cetaceans) may have specifically evolved as an anticancer adaptation for some mismatches between the dynamics of oncogenic processes and natural anticancer adaptations that have occurred during recent evolutionary changes (**Figure 4**). While it is a natural phenomenon that oncogenic processes steadily accumulate in the body with age, pregnancies are unlikely to initiate cancers in an evolutionary stable context. However, mismatches in cancer defense levels can cause oncogenic processes to accumulate at higher rates, enhancing the growth of existing tumors and raising the risk of tipping the balance toward the initiation of uncontrollable metastatic cancers. Thus, after a given age, pregnancy could be associated with a higher probability of premature death due to metastatic cancers. The physiology of fertile women itself is also expected to favor malignant progression because several cancers also depend on hormones (estrogen and progesterone) for their growth (Aktipis et al., 2015; Aktipis, 2016; Atashgaran et al., 2016). Menopause could potentially have evolved as a "natural hormonal therapy" to prevent or alleviate the growth of malignancies before a fatal threshold is reached (Thomas et al., 2019a). If reproduction can contribute to an increase in oncogenic processes later in life, it is however, not the only nor even the most important contributor, and cancers remain common even after menopause and/or in nulliparous women (Gleicher, 2013; Einstein et al., 2015). This emphasizes how cancers must be viewed within multivariate life-history strategies and tradeoffs.

From this hypothesis, further fascinating directions (still debated in humans, e.g., Saadat, 2010) remain to be explored, such as the influence of offspring sex ratio on cancer risk, for example. All else being equal, sons in sexually dimorphic and polygynous species are often more costly to produce or rear than daughters (Bérubé et al., 1996; Whittingham and Dunn, 2000; Gibson and Mace, 2003; Cameron-MacMillan et al., 2007; Rickard et al., 2007). If male embryos more strongly foster the proliferation of existing malignant cells during pregnancy than female embryos, it could be expected that a sex ratio biased in favor of male progeny could, all else being equal, result in a higher overall maternal cancer risk and/or a higher risk of cancer at a younger age. The literature on this topic remains controversial at the moment (e.g., Hsieh et al., 1999; Wohlfahrt and Melbye, 2000; Saadat, 2010).

In addition, it would be particularly relevant to explore whether some of the mammalian species that show no decline in pregnancy rates with increasing age (e.g., white tailed-deer, *Odocoileus virginianus*) (DelGiudice et al., 2007) display a higher risk of cancers or have evolved alternative anti-defenses mechanisms to buffer cancer risk. This approach would also be relevant in ectotherm species that often show no decline (or sometimes even an increase) in fertility with age (e.g., Cayuela et al., 2020). Many studies are currently seeking to identify the biological properties enabling many ectotherm species to escape aging (e.g., Hoekstra et al., 2020 in reptiles). It has been notably highlighted that telomere dynamics can be particularly variable in these species (Hoekstra et al., 2020) with an absence of decline in telomere length with age (e.g., Ujvari and Madsen, 2009 in water python, *Liasis fuscus*). Whether this maintenance of telomere length with age is due to an increased expression of telomerase, which would in turn increased cancer risk is yet to be explored (Olsson et al., 2018) but would constitute a key support for a trade-off between the intensity of reproductive aging and the risk of cancer. Albeit limited, the bunch of empirical evidence compiled so far highlights that the risk of cancer is far from being negligible in ectothermic species such as reptiles (Madsen et al., 2017) which suggest that embracing this research path might be full of promises.

## TRANSMISSIBLE CANCERS AND CANCERS WITH AN INFECTIOUS CAUSATION

At least for dogs and Tasmanian devils, infection by transmissible malignant cells can be viewed as a cost of reproduction. In the vast majority of cases, cancer is not a contagious disease and malignant cells die with their host. However, in at least nine independent cases, cancer cells have evolved the capacity to be horizontally transmitted: one cancer in dogs, two in Tasmanian devils (*Sarcophilus harrisii*), and seven in bivalve species (McCallum et al., 2009; Yonemitsu et al., 2019; Dujon et al., 2020; Garcia-Souto et al., 2022). In dogs, canine transmissible venereal tumor (CTVT) is indeed caused by a sexually transmitted cancer cell line that affects dogs worldwide (Strakova and Murchison, 2014). The tumors are usually localized on the external genitalia (penis and foreskin in males and vulva in females), and it spreads between individuals through sexual intercourse and licking and/or biting the affected areas. These transmissible cell lines appeared between 8,000 and 11,000 years ago (Strakova and Murchison, 2015; Ostrander et al., 2016).

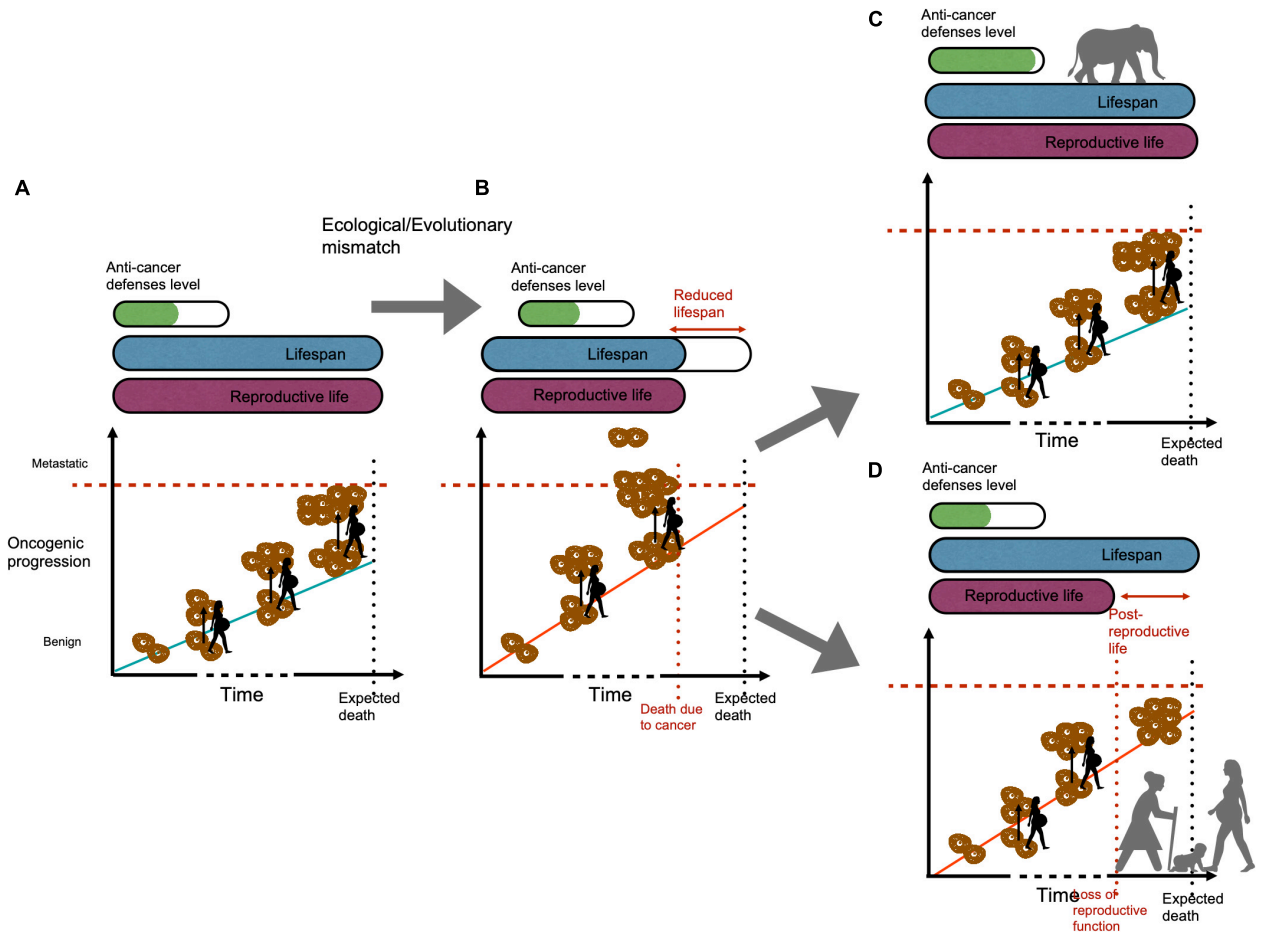


**FIGURE 4 |** Menopause as a possible (transient) adaptation to cancer risk following an evolutionary mismatch. **(A)** When cancer defenses are in alignment with malignant risks, oncogenic processes slowly accumulate through time, and even if pregnancy periods exacerbate their dynamics, the risk of metastatic cancers remains low. **(B)** Following an ecological and/or evolutionary mismatch, cancer defenses may become too weak given the novel cancer risks. Oncogenic processes accumulate rapidly, and this time reproductive episodes exacerbate metastatic cancer risks sooner in life for females, resulting in a short lifespan and a low fitness. From this situation, two evolutionary outcomes are possible, natural selection can (i) favor the evolution of better cancer defenses in these species (i.e., as in large animals such as whales and elephants), coming back to a situation comparable to **(A)**, here **(C)**, or (ii) favor females ceasing their reproduction prematurely to preserve their health and invest their energy/resources for their grandchildren **(D)**. In this later situation, female's fitness is higher than in **(B)** because of inclusive fitness. The **(D)** scenario could in theory be just a transient situation until other cancer defenses are selected (**A,C** examples). Figure modified from Thomas et al. (2019a).

Presumably as the result of this relatively long co-evolution with its hosts, CTVT rarely metastasizes and even displays a regressive stage (Das and Das, 2000; Martincorena et al., 2017). In Tasmanian devils, malignant cells are responsible for two cancers called devil facial tumor disease (DFTD), and devil facial tumor 2 (DFT2). It is not directly sexually transmitted, rather direct contact through biting is required, which frequently occurs during social interactions linked to reproduction. Infected individuals can still mate but they have reduced survival as a consequence of mating. DFTD was discovered in 1996 in northeastern Tasmania and has evolved into at least five clades (Hawkins et al., 2006; Kwon et al., 2020), whereas the second and independently emerged cancer, DFT2, was discovered in southeastern Tasmania at the d'Entrecasteaux Peninsula in 2014; (Pye et al., 2016; James et al., 2019; **Figure 5**). DFTD and DFT2 both induce large ulcerating tumors around the face and jaws, and DFT2 neoplasms are also frequently found in other parts of the body (James et al., 2019). DFTD, for which there is now a lot of information, is clearly fatal, with death usually occurring 9–12 months after the appearance of the first lesions with evidence of some individuals surviving up to 2 years (Wells et al., 2019). In only 25 years, the demographic cost of this transmissible cancer has been devastating, posing a serious conservation threat to the species, which is now classified as "Endangered" by the International Union for Conservation of Nature (Hawkins et al., 2008). While natural selection for less aggressive phenotypes could be expected in devils on the long term, it has not yet been observed, presumably because aggression is a trait associated with increased mating and breeding success in this species (Hamede et al., 2013). In evolutionary terms, it remains more

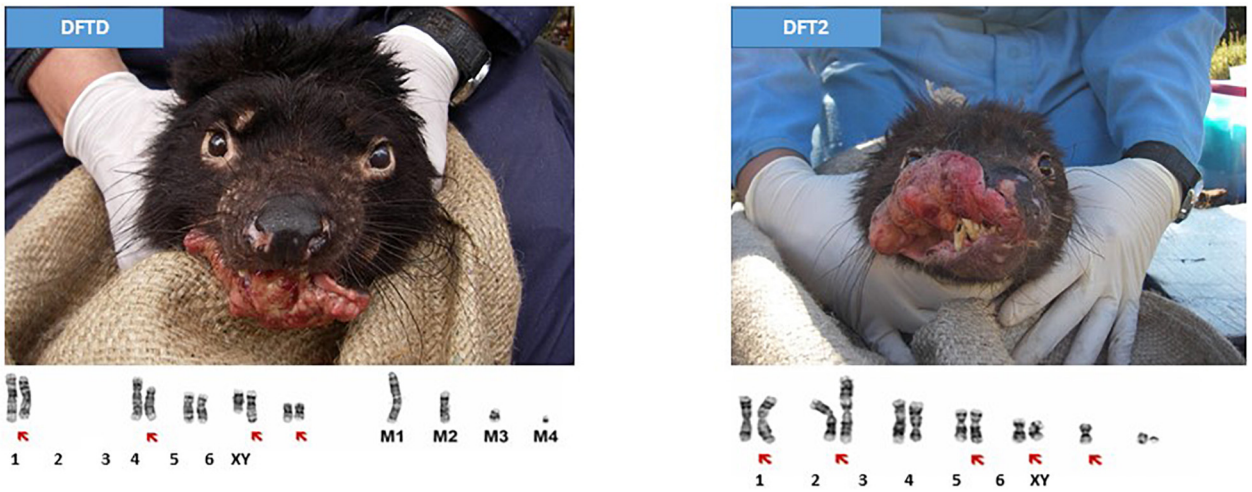


**FIGURE 5 |** Transmissible cancers in Tasmanian devils. Tasmanian devils with DFTD and DFT2 and the respective tumor karyotypes. Red arrows indicate chromosomes carrying cytogenetic abnormalities. The four marker chromosomes found in DFTD are labeled M1 to M4. Karyotypes from Pye et al. (2016).

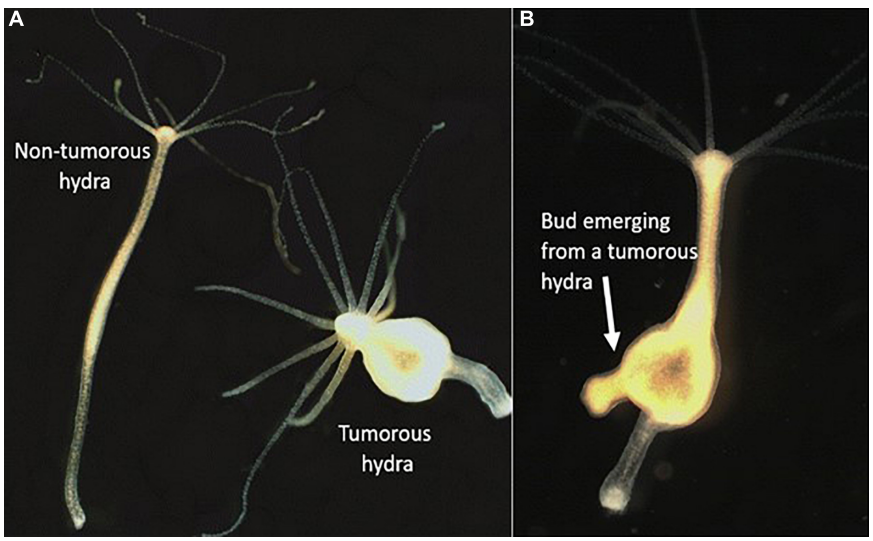


**FIGURE 6 |** Tumors in *Hydra oligactis*. **(A)** Non-tumorous and tumorous hydra. Neoplasia not only severely alter the polyp's body shape, but tumor-bearing individuals also show a shift in their microbiota and display a higher number of tentacles (Domazet-Lošo et al., 2014; Rathje et al., 2020). **(B)** Vertical transmission of tumoral cells in *Hydra oligactis*. After emergence, buds from tumorous hydra are morphologically similar to non-tumorous hydra, but after 4–6 weeks, they develop tumors as their parental polyp (Photo Justine Boutry).

beneficial to reproduce at a cost of developing and dying from DFTD than to remain healthy without reproducing. Interestingly, there is increasing evidence of tumor regressions in the wild, suggesting that devils are adapting to DFTD (Margres et al., 2018, 2020), and that a coexistence of devils and this transmissible cancer may be a long-term enzootic outcome (Wells et al., 2019; Hamede et al., 2020).

Reproduction can also be an opportunity for tumor cells to be vertically transmitted, from parent to offspring. Although rare, transmissions of cancer cells have been observed in humans from mother to fetus (Isoda et al., 2009; Greaves and Hughes, 2018). In addition, neoplastic leukemia cells arising in one monozygotic twin have been shown to transmit to the co-twin via intraplacental anastomoses (Clarkson and Boyse, 1971; Greaves et al., 2003). In the basal metazoan hydra (*Hydra oligactis*), tumor-bearing individuals (**Figure 6**) directly transmit tumoral cells to their offspring when reproducing asexually via budding (Domazet-Lošo et al., 2014). Given the omnipresence of oncogenic processes in multicellular organisms as well as the fact that transmissible cancer cells can have dramatic effects on their host's fitness (e.g., Tasmanian devils), Thomas et al. (2019b) argued that sexual reproduction may have been favored by natural selection over evolutionary time as a radical way to prevent the vertical transmission of cancer cells, since it allows

the offspring's immune system to be more efficient at recognizing and eliminating these cells [but see also Aubier et al. (2020)].

In addition to transmissible malignant cells, several cancers have an infectious causation, and a considerable proportion are transmitted during some step related to reproductive activity. Indeed, pathogens that have been recognized as etiological agents of cancers (e.g., herpes simplex viruses, cytomegalovirus, hepatitis virus, papilloma viruses, human immunodeficiency virus, Eskinazi, 1987) are usually transmitted by genital contact or intimate kissing (Ewald, 2009). For this category of malignancies, cancer risk is not surprisingly associated with increased sexual activity (e.g., Bosch et al., 1996; Hayes et al., 2000; Cooper et al., 2007; Gómez and Santos, 2007).

## CONCLUSION

(1) Reproduction is a central activity for all living organisms, and is also associated with a diversity of costs that need to be considered for a full understanding of the selective landscape in which organisms live and evolve. While decades of research have focused on the identification and implications of these costs, little attention has been devoted to exploring how the schedule of reproductive activities over the life course may also influence malignant cell dynamics and cancer defenses that in turn affect health and survival.

(2) Most ecologists currently consider that reproductive events have little or no impact on oncogenic processes, but this has yet to be properly studied. One must also consider separately the trade-offs that are optimal in a Darwinian logic maximizing reproductive success, from those that are relevant in a public health perspective, for which disease risks occurring after the reproductive period matter too. Further studies would need to explore not only whether there are connections between reproductive costs and the dynamics of malignant processes, but also determine for different species and organs the "shape" of the relationship through time (e.g., linear, exponential, steps with thresholds).

(3) In addition, we need to improve our understanding of the potential interactions between different active evolutionary processes. We can consider birth weight as an example for which it has been established that high values are associated with higher cancer rates later in life. In humans, at least in the past, higher birth weights and/or size have been associated with fitness benefits, especially survival early in life under a wide range of environmental conditions. Certain genes could therefore mediate antagonistic pleiotropy, which is associated with the selection for enhanced birth weight due to survival benefits early in life at the expense of the later increased risk of cancer. Because of the same fitness benefits, this phenomenon could also have a maternal origin, e.g., by modifications to the level of resources available to the fetus during pregnancy and/or by choosing taller men as sexual partners.

(4) Finally, we also need to understand over the long term how these interactions may trigger the evolution of defense mechanisms that prevent and/or alleviate the detrimental effects of malignant cells on the fitness of breeding animals. Whether the directionality of the relationship between reproduction and cancer varies among individuals, populations, or species is poorly understood, but should be explored given that cancer dynamics can sometimes influence reproductive decisions, as is the case in *Drosophila* (see above sections). Research programs addressing these questions are particularly timely since modifications of environmental conditions by human activities have been associated with both alterations in the reproduction biology and increased cancer rates in wild populations. A better understanding of the dynamic interplay between age-specific reproductive activity and the dynamics of oncogenic processes remains a major goal for diverse research areas such as population dynamics, conservation biology, epidemiology, and public health.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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# Reproductive Senescence in Two Lemur Lineages

*Peter M. Kappeler[1,2]*, Leonie Pethig[1,2], Lea Prox[1,2] and Claudia Fichtel[1]*

[1] *Behavioral Ecology and Sociobiology Unit, German Primate Center, Leibniz Institute of Primatology, Göttingen, Germany,*
[2] *Department of Sociobiology and Anthropology, Johann Friedrich Blumenbach Institute of Zoology and Anthropology, Georg-August University, Göttingen, Germany*

The relationship between age and reproductive performance is highly variable across species. Humans and some cetaceans exhibit an extreme form of reproductive senescence in that female reproduction ceases years or even decades before average life expectancy is reached. However, neither the existence of reproductive senescence in some taxa nor its absence in others is fully understood. Comparative data from other long-lived mammals may contribute to a more comprehensive understanding of the evolution of menopause, but data from wild primates, in particular, are scarce. We therefore investigated age-related female reproductive performance in two wild sympatric populations of Malagasy primates: Verreaux's sifakas (*Propithecus verreauxi*) and redfronted lemurs (*Eulemur rufifrons*), which have a maximal longevity of more than 20 years. Based on 25 years of long-term demographic data, we extracted information on reproductive output of 38 female Verreaux's sifakas and 42 female redfronted lemurs. We modeled variation in female reproductive performance and interbirth intervals as a function of age, the number of adult females within a group to account for female competition, and rainfall as a proxy for annual variation in food availability. We also compared our results for these two species with data on captive populations of the same two genera that are buffered from fluctuations in environmental variables. Our analyses disclosed statistical evidence for reproductive senescence in three out of four populations (captive Coquerel's sifakas, wild redfronted lemurs, and captive red lemurs) but not for wild Verreaux's sifakas. Compared to wild populations, reproductive senescence was therefore not less pronounced in captive animals, even though the latter are buffered from environmental adversities. In wild redfronted lemurs, mothers were more likely to give birth in years with more rainfall, but neither the number of co-resident females, nor annual rainfall did predict variation in the probability of giving birth in wild Verreaux's sifakas. Thus, our study contributes valuable comparative information on reproductive senescence in a basal group of primates, and offers insights into the modulating effects of environmental, social and phylogenetic factors on patterns and dynamics of age-specific female reproduction.

**Keywords: reproduction, senescence, interbirth interval, captivity, menopause, lemurs**

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*Sophie Reichert, University of Turku, Turku, Finland*

****Correspondence:***
*Peter M. Kappeler*
*pkappel@gwdg.de*

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## INTRODUCTION

The optimal allocation of reproduction and fertility across the lifespan constitutes a key life-history problem (Stearns, 1976; Roff, 1992; Healy et al., 2019). Theoretical frameworks that predict a trade-off between life-history traits therefore include reproductive senescence, i.e., a decline in reproductive performance, with age (Lemaître et al., 2015, Lemaître and Gaillard, 2017). While some authors have distinguished between aging (the process) and senescence (the outcome), we follow others (e.g., Monaghan et al., 2008; Lemaître et al., 2015) in not making this distinction and using only senescence below. In humans and a few other mammalian species, reproductive senescence terminates in menopause, i.e., the complete cessation of fertility before the average lifespan is reached (Walker and Herndon, 2008; Johnstone and Cant, 2010, 2019; Lieberman et al., 2021). Despite long-held skepticism (Medawar, 1952), there is now compelling evidence for the widespread existence of senescence in a variety of animal species (Monaghan et al., 2008; Nussey et al., 2013). Comparative studies based on long-term data from various wild vertebrate populations have confirmed the presence of early late life trade-offs, suggesting that resource limitation leads individuals to trade somatic maintenance later in life for high allocation to reproduction early in life (Lemaître et al., 2015), and have begun to identify environmental factors that modulate their timing and intensity (Gaillard and Lemaître, 2020). Studying intra- and interspecific variation in longevity and fertility, their complex relationship, as well as the factors modulating them, is therefore crucial for understanding variation in fitness, but our understanding of these processes remains limited, partly because data from very long-lived taxa remain scarce (Gaillard and Lemaître, 2020). Here, we present results of a comparative study exploring these patterns and sources of age-specific female reproduction in relatively long-lived basal primates to contribute to a redressal of this data gap.

As in the vast majority of mammalian species with relatively long lifespan (Lemaître et al., 2020), such as African elephants (Hayward et al., 2014), reproductive rates of female primates are expected to decline with age. Gestation and lactation are energetically demanding, and older female mammals should balance the successful rearing of current offspring against the probability to survive and to produce future offspring (Lemaître and Gaillard, 2017). Indeed, in wild, and thus unprovisioned, chimpanzees and yellow baboons, an age-related decline in female reproductive output was found, but only at relatively old maternal ages and at the same rate as the probability to survive declined (Thompson et al., 2007; Altmann et al., 2010). In some baboon populations, female reproductive activity even ceased before individuals reached their average lifespan (Packer et al., 1998). Further comparisons among six anthropoid (baboons, blue monkeys, capuchins, chimpanzees, gorillas, muriquis) and one lemur species (Verreaux's sifakas) also suggested that reproductive senescence was present – at least in baboons and sifakas statistically significant effects could be demonstrated – but most individuals died before fertility cessation (Alberts et al., 2013). Another comparative study of 13 primate species showed that age-specific fertility declined with increasing age, also in captivity (Caro et al., 1995). Furthermore, in wild but provisioned rhesus macaques, rates of reproductive senescence were disproportionately higher than those of somatic decline (Lee et al., 2021). Finally, female muriquis, blue monkeys, baboons, gorillas and chimpanzees showed longer interbirth intervals (IBIs) in the oldest or youngest age classes, or both, and the oldest females also showed relatively fewer completed IBIs (Campos et al., 2022). Thus, under various conditions of food availability, reproductive senescence has been reported for various primate species. However, the majority of field studies used estimated or statistically modeled age classes and could not draw on known-aged individuals; one of the practical challenges of long-term field studies (Kappeler et al., 2012, 2017).

Whenever longitudinal data on reproductive rates are not available, cross-sectional data on IBI may provide relevant information on reproductive senescence because the probability of prolonged IBIs is predicted to increase with age. The current evidence for this effect is mixed, however. In Barbary macaques, for example, younger mothers wean their offspring indeed significantly earlier than older ones (Paul et al., 1993), indicating that the successful rearing of offspring is age-related, at least in some species (Atsalis and Margulis, 2008). In particular, IBIs were the longest for very young and very old females in this population (Paul et al., 1993). In chimpanzees, IBIs also increased significantly with maternal age, but the effect size was rather small and this effect was probably more due to individual maternal health, as IBI length in this species is closely related to somatic senescence (Thompson et al., 2007). In the comparative study of 13 captive primate species, the majority of correlations between IBIs and female age were negative, but only significant in humans, orangutans and chimpanzees (Caro et al., 1995). Female baboons seemed to experience shorter IBIs with advancing age in this study, but again this correlation was not significant. In wild capuchin, howler and spider monkeys, IBIs were not affected by age, but also not by parity, rank or body weight (Fedigan and Rose, 1995). Among several populations of captive common marmosets, Smucny et al. (2004) found a significant non-linear correlation between maternal age and IBI length. Thus, female primates appear to account for the increasing costs of reproduction with increasing age by adjusting IBIs in some cases.

If the detrimental age-effects cannot be fully compensated by reduced reproductive rates, infant survival may also be compromised. Even if females continue to reproduce at regular intervals throughout their lifetime, advanced maternal age may result in increased rates of miscarriages or negatively affect offspring survival because of reduced birth weights, milk production or other complications (Atsalis and Margulis, 2008; Lemaître and Gaillard, 2017; Comizzoli and Ottinger, 2021). In fact, infant survival in female olive baboons rapidly declined with age, and mothers beyond 21 years of age were more likely to experience a miscarriage (Packer et al., 1998). Yet, although the maternal age effect on offspring survival in semi-free ranging Barbary macaques was statistically not significant, infants of older mothers were more likely to survive than those of younger females (Paul et al., 1993). Thus, the efforts by senescence mothers to reproduce successfully may also be compromised by reduced infant survival.

The social system of a species may also have evolutionary consequences for age-dependent female reproductive strategies. Humans and beluga whales, killer whales, narwhales and short-finned pilot whales have evolved an extreme form of reproductive senescence (i.e., menopause), where the post-reproductive phase can last at least as long as the reproductive phase (Ward et al., 2009; Ellis et al., 2018; Johnstone and Cant, 2019). In these species, menopause appears to be the outcome of kin selection in social systems characterized by appreciable or effective female dispersal by reducing the costs of intergenerational reproductive conflict (Johnstone and Cant, 2019). Reproductive effort can also be compromised by competition with other females independent of age, however (Beehner and Lu, 2013). For example, in Hanuman langurs conception probability was lower and IBIs were longer in groups containing more females (Sommer and Rajpurohit, 1989; Sommer et al., 1992). Thus, the evolution of key life-history traits appears to be also shaped by features of the social system, including patterns of female kinship, mate quality and reproductive competition.

Other factors can modulate reproductive performance on more immediate levels and time scales. In the wild, animals are exposed to natural fluctuations in food availability, rainfall, temperature and parasites, all of which can have instantaneous consequences for the current or next reproductive cycle, and thus, for reproductive rates. These relationships can be investigated by either cross-sectional comparisons across multiple populations or groups, longitudinal contrasts across multiple seasons or years for the same individuals, or by comparing wild and captive populations of the same species or genus. In the present study, we will adopt the latter two approaches, focusing on two lemur genera in the wild and in captivity.

Long-term studies of wild populations are often compromised by practical challenges related to the longevity of the study subjects, which may exceed the academic lifespans of the researchers, and other practical difficulties (see above). In contrast, unmanaged captive populations represent a convenient opportunity to investigate the dynamics of maximal female reproductive rates under conditions of food abundance, veterinary care and reduced parasite exposure. Such effects might be far more significant and easier to detect in species with short lifespans and high reproductive rates (Tidière et al., 2016). Nonetheless, compared to their wild relatives, semi-captive ring-tailed lemurs did not seem to experience reproductive senescence (Lemaître and Gaillard, 2017). However, in two species of captive ruffed lemurs, litter size as well as infant survival follow an inverted U-shaped pattern, with a decreasing point after mothers reached the age of 9 and 11.5 years, respectively (Tidière et al., 2018). In Hanuman langur females, members of free-ranging populations exhibited a later onset of about 10 months of first conception than they do in captivity (Sommer et al., 1992). Similarly, the timing of first reproduction in wild female common marmosets was delayed by several months compared to their captive counterparts (Tardif et al., 2008). Comparisons of reproductive effort between wild and captive populations can therefore provide information about the magnitude of phenotypic plasticity in these traits, offering a benchmark for maximal reproductive effort to which data from populations with various environmental constraints can be compared.

Non-human primates share fundamental features of their life histories with humans (van Schaik and Isler, 2012), so that studying a basal group of primates can contribute valuable comparative information on the evolution of reproductive senescence in general and (human) menopause in particular. Despite their relatively long lifespans, slow development and reproductive rates, long-term field studies of primates have generated valuable demographic data for comparative studies of fundamental questions in life-history evolution (Bronikowski et al., 2011; Morris et al., 2011; Colchero et al., 2016, 2021; Campos et al., 2017, 2022). Lemurs, the endemic primates of Madagascar, have been underrepresented in these comparative studies because of the limited number of long-term study sites (Jolly, 2012; Kappeler and Fichtel, 2012a; Sussman et al., 2012; Wright et al., 2012; Kappeler et al., 2017). They are nonetheless interesting for studies of reproductive senescence because they tend to live longer than most anthropoid primates of similar body mass, with a remarkable maximum longevity of over 30 years for mammals of $< 10$ kg, at least in captivity (Zehr et al., 2014). Lemurs also exhibit impressive interspecific variation in age at first reproduction, litter size and reproductive rates (Kappeler, 1995, 1996; Ross, 1998; Tecot et al., 2013), but patterns and drivers of intraspecific variation have not yet been studied in great detail.

Representatives of two lemur families (Indriidae and Lemuridae) that evolved diurnal activity, group living and single births independently (Kappeler, 1998; Kappeler and Pozzi, 2019), thus resembling anthropoid primates in key life-history features, have been subjected to long-term field studies. The available published information about lemur reproductive senescence can be summarized as follows (for wild mouse lemurs see Rina Evasoa et al., 2018; for captive lemurs: Zehr et al., 2014). First, one study of Milne Edward's sifakas (*Propithecus edwardsi*) revealed that, although no overall effect of age on fertility could be found, offspring survival was significantly decreased in years with below-average rainfall during the lactation period, but the age classification in this study was based on estimates of tooth wear rather than known ages (Wright et al., 2008). Second, in wild Verreaux's sifakas (*P. verreauxi*) at Beza Mahafaly, which were also assigned to one of 5 age classes based on tooth wear analyses, females began reproducing surviving infants with 5 years of age, followed by a sharp increase in reproductive rates over subsequent years and a steady phase of about a decade before a decline in average fertility set in around the age of 18 (Richard et al., 2002). Nonetheless, many females in their early 20 s continued to reproduce regularly, and the oldest mother was estimated to be 28 years old. Controlling for body mass, these sifakas had a greater age at last reproduction than 14 other primate species for which these data were available at the time. Female reproductive performance in this species was also related to unpredictable climate because a severe drought decreased birth rates by almost 20%, and only few offspring born that year survived (Richard et al., 1991, 2000). At Kirindy Forest, the proportion of female Verreaux's sifakas reproducing varied widely across years, and part of this variation was explained by

variation in the number of co-resident females (Kappeler and Fichtel, 2012a). Finally, wild female ring-tailed lemurs, which were also grouped into age-classes, reproduced irrespective of age, but birth rates declined in groups with larger numbers of females and in years with a drought (Gould et al., 2003). In another population, old female ring-tailed lemurs reproduced at slightly, but not significantly, reduced rates, presumably because most of them had already died or disappeared before a clear pattern could have been detected (Ichino et al., 2015).

Additional data from long-term studies of age-specific fertility in the wild are obviously needed to further investigate reproductive senescence and its underlying mechanisms in lemurs. Here, we present such data on sympatric wild Verreaux's sifakas and redfronted lemurs (*Eulemur rufifrons*), offering an opportunity for contrasting the life histories of representatives of independently evolved lineages to identical (fluctuations in) environmental conditions. Both species live in small multi-male, multi-female groups and feed on a broad range of fruits and leaves (Koch et al., 2017). Verreaux's sifakas are slightly larger than redfronted lemurs (2.9 vs. 2.2 kg), their groups are of similar size (6.1 vs. 5.4 individuals), female reproduction begins later (5 vs. 3 years), but both species typically produce singletons and can live well over 20 years (Richard et al., 2000, 2002; Kappeler and Fichtel, 2012a, 2016; Zehr et al., 2014). Despite female philopatry in both species, female Verreaux's sifakas occasionally emigrate into other groups (Kappeler and Fichtel, 2012a), and female redfronted lemurs very rarely succeed in immigrating into other groups after being evicted from their natal one (Kappeler and Fichtel, 2012b). In order to assess the effects of ecological challenges on female reproduction, we compare our results with data from closely related captive *Propithecus* and *Eulemur* species, for which the Duke Lemur Center (DLC) (Durham, NC, United States) has accumulated an impressive data set under uniform captive conditions (Zehr et al., 2014).

The main aims of our study are therefore to evaluate female reproductive output and IBIs in two sympatric wild lemur populations as a function of age, the number of adult females (to account for female competition) as well as rainfall as a proxy for variation in food availability. To further investigate the relationship between ecology and life-history dynamics, we compare data on wild and captive populations of the same two genera to account for annual environmental variation and its consequences for reproductive senescence in the unpredictable environment of Madagascar. In line with the relevant evolutionary theory, we expected reproductive senescence (i) to occur in these relatively long-lived small mammals, and (ii) to be less pronounced in captive animals because they are buffered from natural hazards.

# MATERIALS AND METHODS

The data presented in this study come from two sources. First, we collected demographic and life-history data from sympatric populations of Verreaux's sifakas and redfronted lemurs at Kirindy Forest, western Madagascar, a dry deciduous forest exposed to pronounced climatic seasonality (Kappeler and Fichtel, 2012a). Beginning in 1995 (sifakas) and 1996 (redfronted lemurs), members of several adjacent groups of both species, including immigrants and newborns, have been regularly captured, measured and individually marked with Passive Integrated Transponder (PIT) tags and unique nylon collars. One individual per group has been equipped with a radio collar to facilitate multiple censuses per week during which each group's composition, including the number of co-resident adult females, has been recorded. Birth dates of newborn infants are therefore known with a precision of a few days; in most cases we know the exact birthday. Ages of individuals at the beginning of the study could be determined with an accuracy of a year because both species are strict annual breeders and juveniles of different ages clearly differ in body mass from each other; ages of adults during first captures were estimated based on patterns of tooth wear. We therefore created two data sets for the analyses of age-dependent reproductive rates of these two species: dataset A contains only individuals with exactly known ages; dataset B also includes females that were adults at the beginning of the study and whose ages have been estimated. Finally, because climatic data were not collected regularly during the early years of the study, we used published rainfall data from the CHIRPS (n.d.) data base to assess interannual variation on precipitation.

Second, in February 2022 we extracted life-history data from the published records of the Duke Lemur Center (DLC; Zehr et al., 2014), which houses captive colonies of sister species of the two species studied at Kirindy. Because *P. verreauxi* and *E. rufifrons* are not kept at the DLC, we extracted reproductive data (i.e., age at first reproduction, IBIs, age at all subsequent births) for Coquerel's sifaka (*P. coquereli*) and red lemur (*E. rufus*) females of known ages. Both species are similar to *P. verreauxi* and *E. rufifrons* in body size, social system and also inhabit the dry deciduous forests of western Madagascar in the wild (Mayor et al., 2004; Razafindramanana et al., 2020).

## Statistical Analyses

### Comparison of Age at First and Last Reproduction Between Wild and Captive Populations

We compared the age at first reproduction of females with known age between the captive and wild populations using a Mann–Whitney U-test. Age at last reproduction was compared between captive and wild lemurs with known and estimated age using a Kruskal–Wallis test. Since age was estimated for only three captive red lemur females, we did not include them in this comparison.

### Age-Specific Reproductive Performance

To estimate the effect of age on the likelihood of giving birth to an offspring, we fitted Generalized Linear Mixed Models (GLMM; Baayen, 2008) with binomial error structure and logit link function, with giving birth as the binary response ("yes" or "no"). For wild lemurs, we included as fixed effects, female's age, the number of adult females present in the month of birth, and annual cumulative rainfall as control factors. Since reproductive senescence is a within-individual process, we separated within-individual aging patterns from between-individual heterogeneity, i.e., selective appearance or disappearance of individuals, by

including longevity or age at first or last reproduction as additional control factors (van de Pol and Verhulst, 2006; Nussey et al., 2008). We fitted this model on each of the two data sets (A and B) for wild lemurs, one on all females including those for which the exact age was not known and the one including only females with known age. Longevity was included in the model including all females, whereas in the subset of females with known age, we included age at first and last birth to account for both, selective appearance and disappearance (van de Pol and Verhulst, 2006; Nussey et al., 2008). As random effects, we included female and group identity. For wild lemurs we included the random slopes of female's age, the number of adult females and rainfall within mothers and groups. Originally, we also included correlations between random slopes and intercepts, but as the models did not converge, we had to exclude them again. As information on group composition as well as group identity was not available for data on captive lemurs, we included as fixed effects only female's age and longevity because birth rates might have been influenced by husbandry decisions. Female's age within the random slope of female's identity was included as random factor. In Coquerel's sifakas, we had to remove the correlation between random slopes and intercepts, as the models did not converge.

For each data set, we fitted three models, one including only "age," one including "age and quadratic age," and a threshold model (see **Supplementary Material** for a detailed description of the threshold model). Most, except three models, including age and quadratic age did not reveal a negative estimate for quadratic age, thus, not matching the expected inverted U-shape. Therefore, we report these models only in the **Supplementary Material** (**Supplementary Tables 1–4**). For models where the estimate for quadratic age was negative, we compared AIC values between the model including only age and age as well as quadratic age, and present the models with a delta AIC $< 2$ in the main text. Since the results of all threshold models, except one, revealed negative estimates for both, the lower threshold age as well as the upper threshold age, not matching the roughly expected inverted U-shape, we do not report model outputs. Finally, we explored whether females experienced reproductive completion. Females, that lived longer than the mean IBI + 2*SD after having given birth to the last live infant were considered as having completed reproduction (Alberts et al., 2013).

We compared all models to a null model, comprising all random and fixed effects except for age, with a likelihood ratio test (Schielzeth and Forstmeier, 2009). This full-null model serves to avoid "cryptic multiple testing" (Forstmeier and Schielzeth, 2011). Sample sizes for the reproductive output models can be found in **Tables 1**, **4**.

### Interbirth Intervals

To determine whether female age has an effect on IBIs, we fitted Generalized Linear Mixed Models with a Gamma-distribution and a log link. For the two wild populations, we used IBIs as the response and female's age, number of adult females present at birth, annual cumulative rainfall and longevity or age at first and last birth as fixed effects, as well as individual and group identity as random effects. For Verreaux's sifakas we included the random slopes of age, the number of adult females and rainfall within mothers as well as age, longevity and rainfall within groups without correlations between random slopes and intercepts. For wild redfronted lemurs, we included the random slopes of age, the number of adult females and rainfall within mothers as well as age, the number of adult females, longevity and rainfall within groups. As in the reproductive output models, we estimated a model including only "age" and one including "age" and "quadratic age," respectively. Similar to the reproductive output models, we compared AIC values of models including age or age and quadratic age, when the estimate of quadratic age was positive. In addition, all full models were compared to a null model, comprising all random and fixed effects except for age, and we checked for overdispersion, which revealed that all models were highly underdispersed. Therefore, we did not fit additional threshold models. Sample sizes for IBI models can be found in **Table 1**. For the two captive populations, we did not estimate whether IBIs were influenced by mothers age because some females were put on contraception due to husbandry decisions.

Analyses were conducted using R (version 4.1.0; R Core Team, 2020), applying the function glmer and lmer from the package "lme4" (version 1.1-21; Bates et al., 2015). We centered all quantitative predictors to a mean of zero and a standard deviation of one before including them into the models, to facilitate model convergence. All theoretically identifiable random slopes were included to avoid Type I errors (Barr et al., 2013). Confidence intervals were obtained for all models by means of parametric bootstraps using the function "bootMer" of the package "lme4," applying 1000 parametric bootstraps. We checked for collinearity by determining Variance Inflation Factors (VIF; Dobson and Barnett, 2018) for a standard linear model without random effects using the package "car" (version 3.0.11; Field, 2005). VIF values were smaller than 1.9 in all cases. We estimated model stability by dropping levels of the random effect one at a time from the data set and compared the obtained estimates to the estimates obtained for the full data set. This revealed that all models exhibited good stability.

## RESULTS

### Wild Verreaux's Sifakas

In total, 38 wild sifaka females gave birth to 225 singleton infants across 25 years. All females, except three, gave birth to more than one infant during our study. The exact age was known for 13 females, born 1995 or later, who had an age at first reproduction of 5.56 ± 1.06 (mean ± SD; **Table 1**) years. Of these 13 females, seven had already died at the time of this study. These females had an age at last reproduction of 11.65 ± 4.39 years. Of the other females ($N = 25$), for which we estimated age, one was still alive, and the remaining 24 females had an age at last reproduction of 11.97 ± 4.88 years. Only two of these females lived longer than the mean IBI + 2*SD after having given birth to the last live infant (**Figure 2A**). They were 11.98 and 21.08 years old, respectively. Two females that were still alive also experienced a long IBI, but they were rather young, about 8 years each,

**TABLE 1 |** Overview of life-history milestones and sample sizes of wild Verreaux's and captive Coquerel's sifakas.

| | **Wild Verreaux's sifakas** | | **Captive Coquerel's sifakas** | |
|---|---|---|---|---|
| | **Known age (*N* = 13)** | **Estimated age (*N* = 25)** | **Known age (*N* = 35)** | |
| Age 1st reproduction (years; mean ± SD) | 5.56 ± 1.06 | – | 5.85 ± 1.96 | |
| | Females with known reproductive lifespan (*N* = 7) | Females with estimated reproductive lifespan (*N* = 24) | Females with known reproductive lifespan (*N* = 16) | |
| Age last reproduction (years; mean ± SD) | 11.65 ± 4.39 | 11.97 ± 4.88 | 11.21 ± 4.20 | |
| | Females with known reproductive lifespan (*N* = 7) | All females (*N* = 38) | Females with known reproductive lifespan (*N* = 16) | All females (*N* = 35) |
| Total number of infants (mean ± SD) | 5.86 ± 3.49 | 5.92 ± 3.83 | 6.77 ± 3.84 | 5.6 ± 3.88 |
| Interbirth interval (years; mean ± SD) | 1.25 ± 0.44 | | 1.21 ± 0.7 | |

indicating that these long IBIs might have been due to other reasons than reproductive completion.

Overall, Verreaux's sifaka females gave birth to 5.92 ± 3.83 infants during their entire lifespan, with a range of 1 and 16 infants. Focusing on females of known reproductive lifespan only ($N$ = 7), we found that they produced on average 5.86 ± 3.49 infants. The average IBI for all females was 1.25 ± 0.44 years (**Table 1**). Following 74% of births, females gave birth again after about 1 year later, in 22% of cases they gave birth again about 2 years later, and only in two cases (2%) females gave birth again after 3 years (**Figure 1C**). The latter two cases occurred toward the end of the reproductive lifespan (i.e., at 9 and 11 years, respectively).

In the model including all females, the probability of giving birth was independent of female's age, the number of co-resident females, annual rainfall and longevity (**Figure 1A** and **Table 2A**). Including quadratic age in the model did not result in a better fit (AIC: age = 312, age and quadratic age = 318). In the subset of females with known age, the probability of giving birth was also independent of female's age, the number of co-resident females, annual rainfall, and age at first or last birth (**Table 2B**). Adding quadratic age did not improve the model fit (AIC: age = 127, age and quadratic age = 133).

The models on variation in IBIs including age revealed a better fit than those including age and quadratic age (AIC: all females: age = 155, age and quadratic age = 159; females with known age: age = 61, age and quadratic age = 67). Variation in IBIs was neither predicted by mother's age, nor number of adult females in a group, rainfall or longevity (**Table 2C**). Similarly, in the subset of females with known age, variation in IBIs was neither predicted by mother's age, number of adult females in a group, rainfall nor age at first or last birth (**Table 2D**). Both models where highly under-dispersed (all females: dispersions parameter = 0.074, $p$ = 1, females with known age: dispersions parameter = 0.078, $p$ = 1), so that results should be treated cautiously. Hence, our analyses revealed no evidence for wild Verreaux's sifakas to experience reproductive senescence, neither in the probability of giving birth, nor in terms of extending their IBIs.

## Captive Coquerel's Sifakas

In total, 35 captive sifaka females gave birth to 196 singleton infants across 41 years. The exact age was known for all females. Age at first reproduction was on average 5.85 ± 1.96 years, which did not differ between captive and wild sifakas (Mann–Whitney U-test: $z$ = 0.034 $P$ = 0.972; **Table 1**). Four females gave birth only once, three of them were alive at the time of the census, and one had already died. Thirty-one females gave birth to more than one infant. Of those, 15 females were still alive at the time of the census and 16 had already died. The latter subsample had an age at last birth of 13.21 ± 4.20. Age at last reproduction did not differ among captive and wild sifakas with known or estimated age (Kruskal–Wallis: $\chi^2$ = 0.317, $P$ = 0.853). After having given birth to the last live infant, six females lived longer than the mean IBI + 2*SD (**Figure 2B**). One of them died at an age of 11.91 years and gave birth to the last live infant 4.99 years before. The other five females were still alive, two were 5 and 7 years old, and three between 9 to 13 years, suggesting that the latter three ones might have already experienced reproductive completion. However, since we do not know whether some females failed to give birth because of management decisions, these IBIs should be treated with caution. Therefore, we did not estimate whether variation in IBIs covaried with mother's age.

Overall, Coquerel's sifaka females gave birth to an average of 5.6 ± 3.88 infants, with a range between 1 and 13 infants (**Table 1**). Mothers of known reproductive lifespan ($N$ = 16) produced on average 6.77 ± 3.83 infants. The average IBI was 1.21 ± 0.7 years and they did not exhibit such a clear annual pattern as wild sifakas (**Table 1** and **Figure 1C**). The model fitting the probability of giving birth in captive sifakas revealed a better fit when was age included in comparison to age and quadratic age (AIC: age = 270, age and quadratic age = 273). Older females were less likely to give birth, and birth rates were

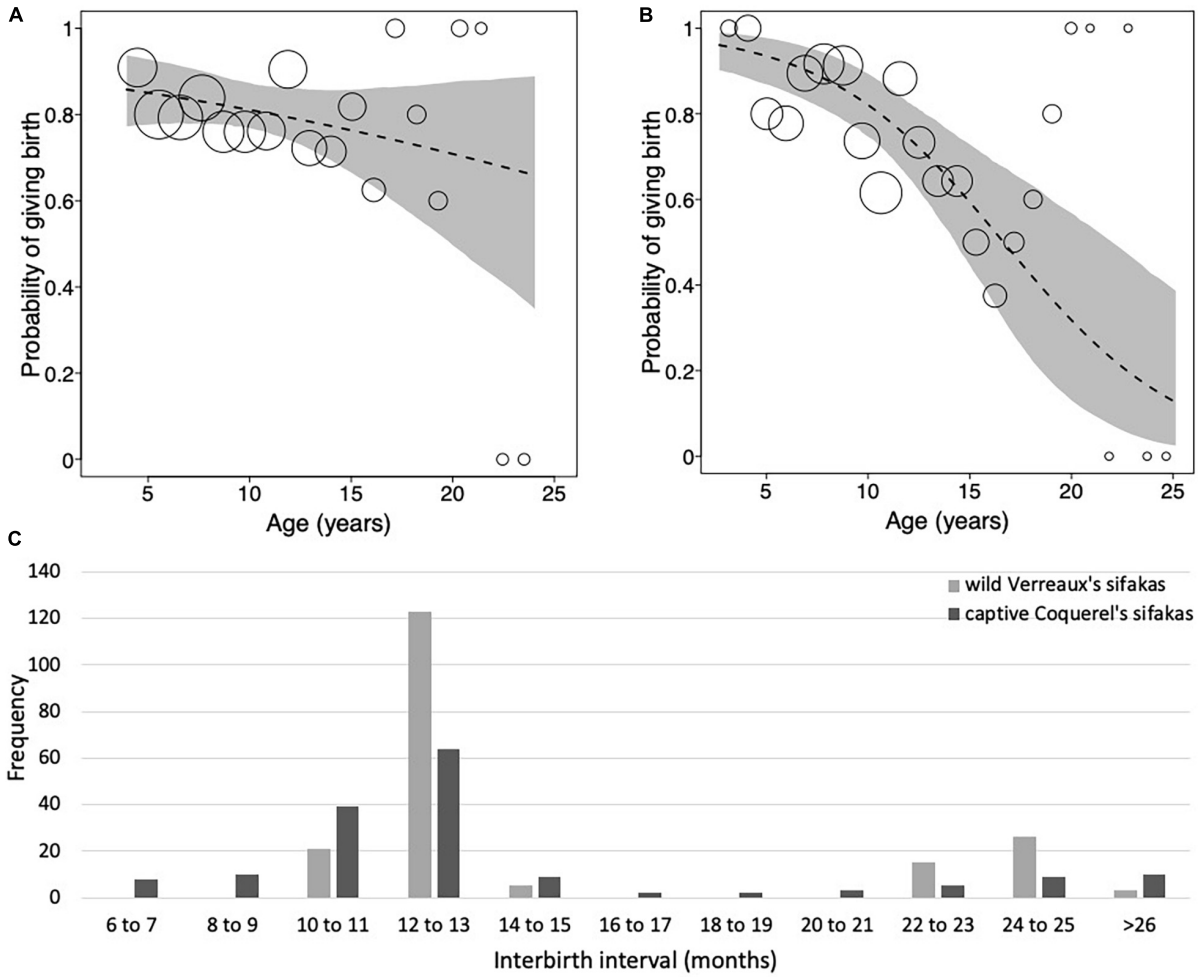


**FIGURE 1 |** Probability of giving birth as a function of mother's age for **(A)** wild Verreaux's sifakas and **(B)** captive Coquerel's sifakas. **(C)** Frequency distribution of interbirth intervals of wild Verreaux's sifakas (light gray; *N* = 193) and captive Coquerel's sifakas (dark gray; *N* = 161). Dashed lines represent the regression lines, gray polygons represent the 95% confidence intervals, and dots represent the number of observations with the area of dots being proportionate to the number of observations per binned age (Verreaux's sifakas: range 2–33, Coquerel's sifakas: 2–26). Note that the dots in panels **(A,B)** are not to the same scale.

influenced by longevity only by trend (**Figure 1B** and **Table 3**). Hence, captive Coquerel's sifakas appear to have experienced reproductive senescence.

## Wild Redfronted Lemurs

In total, 42 redfronted lemur females gave birth to 201 singleton infants, and one female gave birth to twins across 25 years. Eight females gave birth to only one infant during our study period. The exact age was known for 26 females, who had an age at first reproduction of 3.59 ± 0.82 years (**Table 4**). Of these 26 females born in 1996 or later, 24 had already died by the time of this study. They had an age at last reproduction of 8.22 ± 4.22 years. Of the other females (*N* = 16), for which we estimated their age, one was still alive, and the remaining 15 females had an age at last reproduction of 8.24 ± 4.67 years. After having given birth to the last live infant, seven females lived longer than the mean IBI + 2*SD (**Figure 3A**). Their age ranged between 5.75 and 20.54 years, with only two females being older than the mean age of last reproduction. They were 18.50 and 20.54 years old at the time of death and gave birth to their last infant at an age of 16.07 and 18.04 years, respectively. Hence, these two mothers may have completed reproduction but were reproductively active until a very old age.

Overall, redfronted lemur females gave birth to 4.83 ± 3.52 infants, with a range between 1 and 17 infants. Females of known reproductive lifespan (*N* = 24), produced on average 4.75 ± 3.19 infants. The average IBI was 1.20 ± 0.50 years (**Figure 4C** and **Table 4**). Following 83% of births, females gave birth again after about 1 year later, in 14% of cases they gave birth again about 2 years later, and only in five cases (3%) females gave birth again after 3 years or more. One case occurred already at age of 3.99 years, whereas the other four cases occurred toward the end of the reproductive lifespan (i.e., at 8, 11, 18 and 15 years, respectively). Hence, wild redfronted lemurs might have experienced an effect of reproductive senescence, but average IBIs did not change with age (**Figure 4C**).

**TABLE 2 |** Results of the models in wild Verreaux's sifakas on **(A)** the probability of giving birth for all females, **(B)** for female's with known age, and variation in interbirth intervals **(C)** for all females and **(D)** for females with known age.

| Model | Full-null model comparison | Term | Estimate | SE | *P* |
|---|---|---|---|---|---|
| **(A)** Probability of giving birth | $\chi^2$ = 1.627 | Intercept | 1.442 | 0.163 | [a] |
| | df = 1 | Age | –0.240 | 0.189 | 0.205 |
| Age | $p$ = 0.202 | N adult females | –0.087 | 0.154 | 0.573 |
| | | Longevity | –0.043 | 0.191 | 0.823 |
| All females | | Rainfall | –0.117 | 0.232 | 0.612 |
| **(B)** Probability of giving birth | $\chi^2$ = 0.034 | Intercept | 1.319 | 0.291 | a |
| | df = 1 | Age | 0.066 | 0.358 | 0.854 |
| Age | $p$ = 0.855 | N adult females | –0.028 | 0.480 | 0.954 |
| | | Age at first birth | –0.152 | 0.295 | 0.606 |
| Females with known age | | Age at last birth | 0.121 | 0.370 | 0.743 |
| | | Rainfall | 0.438 | 0.282 | 0.121 |
| **(C)** Inter-birth interval | $\chi^2$ = 0.037 | Intercept | 0.268 | 0.050 | [a] |
| | df = 1 | Age | 0.007 | 0.037 | 0.852 |
| Age | $p$ = 0.848 | N adult females | 0.051 | 0.049 | 0.303 |
| | | Longevity | 0.061 | 0.049 | 0.215 |
| All females | | Rainfall | –0.068 | 0.046 | 0.142 |
| **(D)** Inter-birth interval | $\chi^2$ = 0.051 | Intercept | 0.243 | 0.082 | [a] |
| | df = 1 | Age | –0.014 | 0.062 | 0.817 |
| Age | $p$ = 0.821 | N adult females | 0.059 | 0.044 | 0.183 |
| | | Age at first birth | 0.016 | 0.069 | 0.811 |
| Females with known age | | Age at last birth | 0.098 | 0.087 | 0.259 |
| | | Rainfall | –0.075 | 0.048 | 0.115 |

[a]*Not shown as has no meaningful interpretation.*

The model including age provided a better fit than the model including age and quadratic age (AIC: all females: age = 259, age and quadratic age = 263). The probability of giving birth was influenced by female's age and rainfall (**Figure 4A** and **Table 5A**). Older females were less likely to give birth and females were more likely to give birth in years with more rainfall. Longevity did not influence the probability of giving birth. In the subset of females with known age, the probability of giving birth was also better predicted by the model including age than by the one including age and quadratic age (AIC: all females: age = 189, age and quadratic age = 194). Older females were less likely to give birth, and females were more likely to give birth when more adult females were in the group as well as in years with more rainfall (**Table 5B**). Age at first and last infant did not co-vary with the probability of giving birth.

In both data sets (all females, females with known age), variation in IBIs was better explained by the model including age and quadratic age than only age (AIC: all females: age = 158, age and quadratic age = 129, females with known age: age = 125, age and quadratic age = 121). In the model including all females, interbirth length was not influenced by female's age, number of adult females, longevity but by rainfall. IBIs were longer in years with less rainfall (**Table 5C**). In the data set including only females with known age, interbirth length was influenced by none of the factors, and rainfall influenced the length of IBIs only by trend (**Table 5D**). Since both models were highly under-dispersed (all females: dispersions parameter = 0.09, $p$ = 1, females with known age: dispersions parameter = 0.11, $p$ = 1) and only by trend significantly different from the null model (**Tables 5C,D**), results should be treated with caution.

## Captive Red Lemurs

In total, 35 captive red lemur females gave birth to 130 singleton infants and five mothers gave birth to 11 twins across 56 years. Six females gave birth to only one infant. The exact age was known for all females, which had an age at first reproduction of 3.64 ± 3.493 years. In comparison to wild redfronted lemurs, captive red lemurs were on average slightly older at first reproduction (Mann–Whitney U-test: $z$ = 2.456, $p$ = 0.014). Of these 35 females, 17 are known to have already died at the time of this study. These females had an age at last reproduction of 6.91 ± 5.81 years. Age at last reproduction did not differ between captive and wild redfronted lemurs with known or estimated age (Kruskal–Wallis: $\chi^2$ = 2.037, $P$ = 0.361). After having given birth to the last live infant, 18 females lived longer than the mean IBI + 2*SD (**Figure 3B**). They were between 6.79 and 32.67 years old (mean = 20.02, SD = 7.66), with almost all females being older than the age of last reproduction of females with known reproductive lifespan, i.e., 6.91 ± 5.81 years. Since we do not know whether some females failed to give birth because of management decisions, these IBIs should be treated with caution. Therefore, we did not estimate whether variation in IBIs covaried with mother's age.

Overall, red lemur females gave birth to 4.34 ± 4.65 infants, with a range between 1 and 19 infants. Females of known reproductive lifespan ($N$ = 17) produced on average 3.71 ± 4.01 infants. The average IBI was 1.39 ± 1.11 years

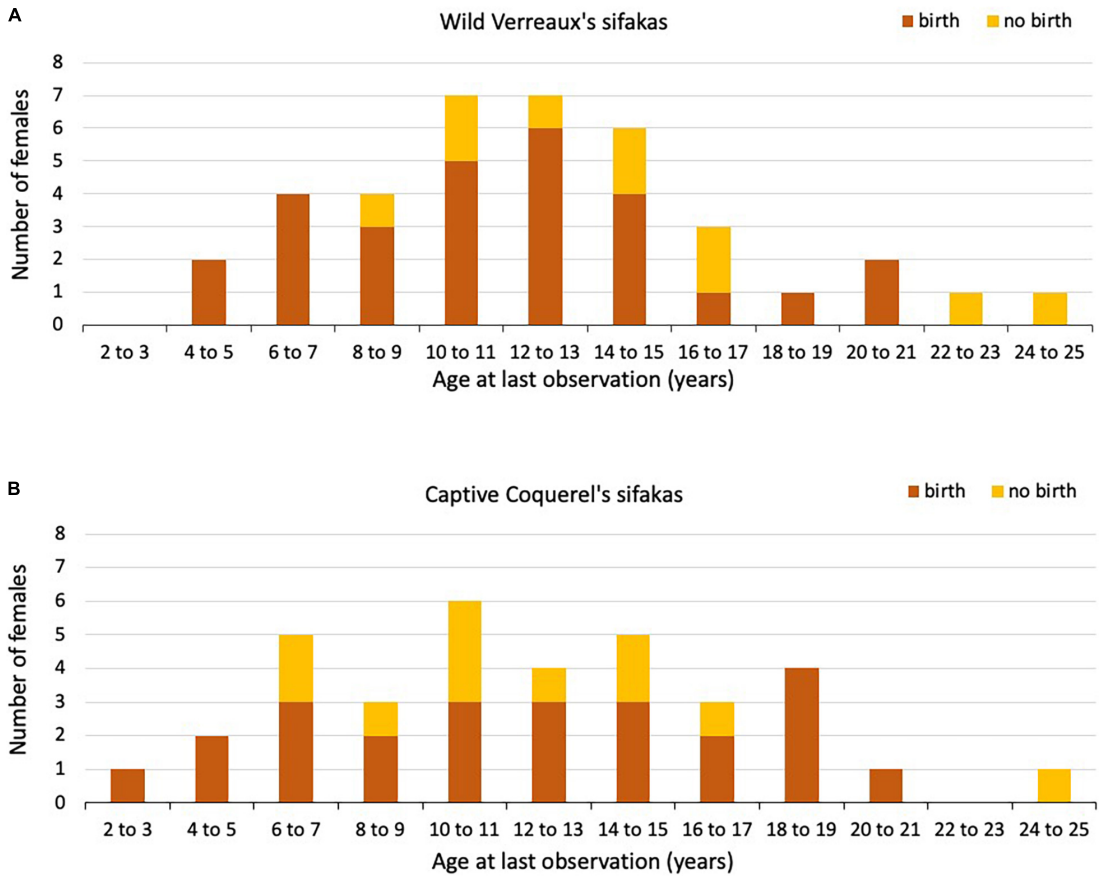


**FIGURE 2 |** Number of **(A)** wild Verreaux's sifaka and **(B)** captive Coquerel's sifaka females that gave birth (orange) or not (yellow) in their last year of life or observation (age in years).

(**Figure 4C** and **Table 4**). The model on the probability of giving birth in red lemurs including age revealed a better fit than the one including age and quadratic age (AIC: age = 330. age and quadratic age = 354). We found an effect of female's age but not of longevity on the probability of giving birth, with older females being less likely to give birth (**Figure 4B** and **Table 6**). Hence, captive red lemurs might also have experienced an effect of reproductive senescence.

## DISCUSSION

Previous research indicated that lemurs do not experience any form of reproductive cessation and thus, are principally capable of reproducing until death (Wright et al., 2008), whereas other studies revealed reproductive senescence late in life (Alberts et al., 2013). The present analyses revealed strong evidence that females in three out of four populations experience reproductive senescence, thereby offering a basis for an exploration of the sources and dynamics of age-specific female reproduction in independently evolved lineages under different environmental conditions.

**TABLE 3 |** Results of the models in captive Coquerel's sifakas on the probability to give birth.

| Model | Full-null model comparison | Term | Estimate | SE | *P* |
|---|---|---|---|---|---|
| Probability of giving birth | $\chi^2$ = 19.080 | Intercept | 1.406 | 0.248 | [a] |
| | df = 1 | Age | –0.979 | 0.236 | **0.000** |
| Age | $p$ < 0.001 | Longevity | 0.493 | 0.289 | 0.087 |

*Significant effect in bold.* [a]*Not shown as has no meaningful interpretation.*

In line with our first prediction, we did find evidence for reproductive senescence in three populations of these relatively long-lived small mammals. In wild redfronted lemurs, captive red lemurs and captive Coquerel's sifakas, the probability of giving birth decreased with mother's age, but it was unaffected by maternal age in wild Verreaux's sifakas. While the pattern observed in the first three of our study populations corresponds to the pattern also observed in about 70% of mammalian species to date (Lemaître et al., 2020), the absence of evidence for reproductive senescence in Verreaux's sifaka is intriguing, also because it only partly corroborates results of previous studies with data from another population of this species, which reported reproductive senescence (Alberts et al., 2013) but no change in IBIs with age (Campos et al., 2022). Thus, populations may vary in patterns of reproductive senescence

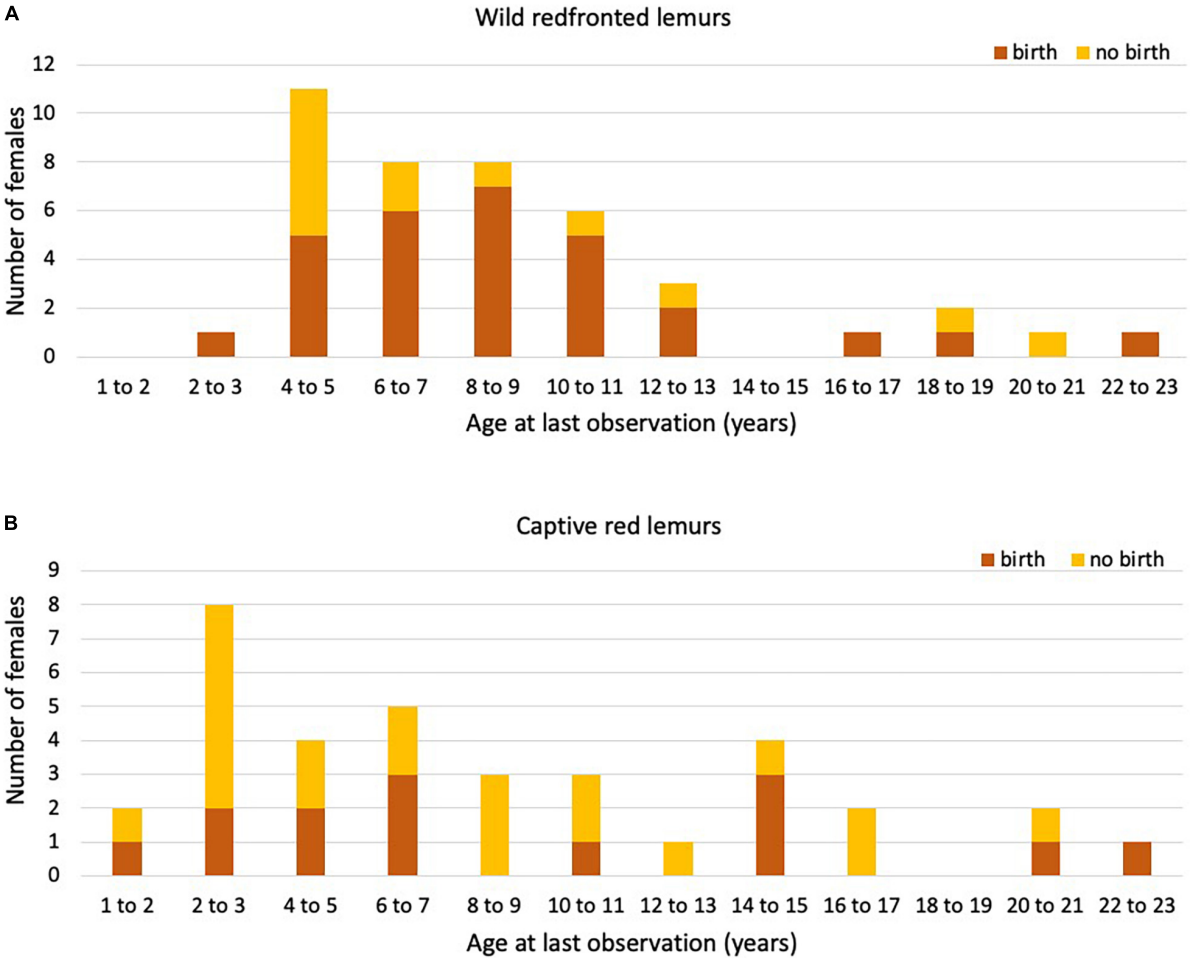


**FIGURE 3 |** Number of **(A)** wild redfronted and **(B)** captive red lemur females that gave birth (orange) or not (yellow) in their last year of life or observation (age in years).

**TABLE 4 |** Overview of life-history milestones and sample sizes of wild redfronted lemurs and captive red lemurs.

| | **Wild redfronted lemurs** | | **Captive red lemurs** | |
|---|---|---|---|---|
| | **Known age (*N* = 26)** | **Estimated age (*N* = 16)** | **Known age (*N* = 35)** | |
| Age 1st reproduction (years; mean ± SD) | 3.59 ± 0.82 | – | 3.64 ± 3.49 | |
| | Females with known reproductive lifespan (*N* = 24) | Females with estimated reproductive lifespan (*N* = 15) | Females with known reproductive lifespan (*N* = 17) | |
| Age last reproduction (years; mean ± SD) | 8.22 ± 4.22 | 8.24 ± 4.67 | 6.91 ± 5.81 | |
| | Females with known reproductive lifespan (*N* = 24) | All females (*N* = 42) | Females with known reproductive lifespan (*N* = 17) | All females (*N* = 35) |
| Total number of infants (mean ± SD) | 4.75 ± 3.19 | 4.83 ± 3.52 | 3.71 ± 4.01 | 4.34 ± 4.65 |
| Interbirth interval (years; mean ± SD) | 1.20 ± 0.50 | | 1.39 ± 1.11 | |

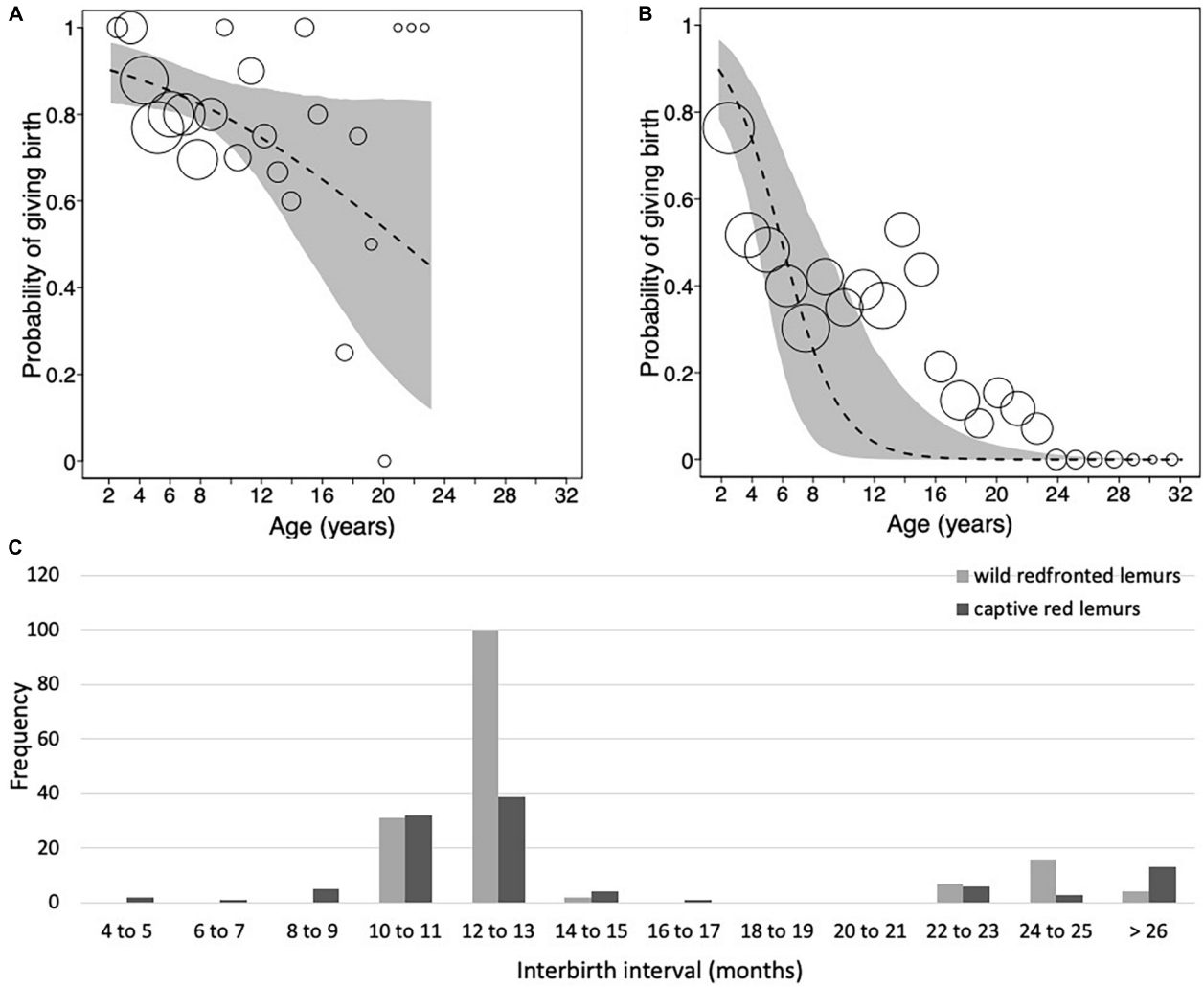


**FIGURE 4 |** Probability of giving birth as a function of mother's age for **(A)** wild redfronted lemur and **(B)** captive red lemur. **(C)** Frequency distribution of interbirth intervals of wild redfronted lemurs (light gray; *N* = 132) and captive red lemurs (dark gray; *N* = 103). Dashed lines represent the regression lines, gray polygons represent the 95% confidence intervals, and dots represent the number of observations with the area of dots being proportionate to the number of observations per binned age (redfronted lemurs: range 2–23, red lemurs: 2–26). Note that the dots in panels **(A,B)** are not to the same scale.

and/or different components of reproductive senescence may vary independently.

This outcome across populations rejects our second prediction, stating that reproductive senescence might be less pronounced in captive animals because they are buffered from external adversities. With respect to the relative importance of age in modulating reproductive rates, we found that neither the number of co-resident females, nor annual rainfall did predict variation in the probability of giving birth in wild Verreaux's sifakas either. In wild redfronted lemurs, in contrast, mothers were more likely to give birth in years with more rainfall. Finally, birth rates did not co-vary with any proxy indicating potential selective appearance or disappearance in the population. i.e., longevity or age at first or last reproduction (van de Pol and Verhulst, 2006; Nussey et al., 2008). Only in captive Coquerel's sifakas longevity co-varied by trend with birth rates, suggesting that longer-lived females might have been more efficient at giving birth throughout their life.

## Prediction 1: Age Effects

Precise age estimates of relatively long-lived mammals like these lemurs require year- or even decade-long investment into long-term field studies. Because such studies are rare or challenging to sustain long enough (Kappeler et al., 2012, 2017), age has also been estimated from tooth wear or other morphological traits, or individuals have been grouped into coarse age classes. While estimates are clearly a less preferred, albeit often unavoidable option, the magnitude of the discrepancies between the two approaches remain poorly known. Our study offered a rare opportunity to compare classes of females of the same populations with either precisely known or estimated ages. In both Verreaux's sifakas and redfronted lemurs, age at first or last

**TABLE 5 |** Results of the models in wild redfronted lemurs on **(A)** the probability of giving birth for all females, **(B)** for female's with known age, and variation in interbirth intervals **(C)** for all females and **(D)** for females with known age.

| Model | Full-null model comparison | Term | Estimate | SE | *P* |
|---|---|---|---|---|---|
| **(A)** Probability of giving birth | $\chi^2$ = 4.682 | Intercept | 1.549 | 0.201 | [a] |
| | df = 1 | Age | –0.492 | 0.235 | **0.037** |
| Age | $p$ = 0.030 | N adult females | 0.454 | 0.273 | 0.096 |
| | | Longevity | 0.294 | 0.261 | 0.260 |
| All females | | Rainfall | 0.565 | 0.172 | **0.001** |
| **(B)** Probability of giving birth | $\chi^2$ = 7.107 | Intercept | 1.439 | 0.258 | [a] |
| | df = 1 | Age | –0.726 | 0.284 | **0.011** |
| Age | $p$ = 0.008 | N adult females | 0.794 | 0.335 | **0.018** |
| | | Age at first birth | 0.303 | 0.223 | 0.175 |
| Females with known age | | Age at last birth | 0.445 | 0.295 | 0.131 |
| | | Rainfall | 0.585 | 0.247 | **0.018** |
| **(C)** Inter-birth interval | $\chi^2$ = 5.218 | Intercept | 0.246 | 0.045 | [a] |
| | df = 2 | Age | 0.073 | 0.060 | 0.223 |
| Age and quadratic age | $p$ = 0.077 | $Age^2$ | –0.133 | 0.090 | 0.141 |
| | | N adult females | 0.018 | 0.043 | 0.669 |
| | | Longevity | 0.036 | 0.039 | 0.356 |
| All females | | Rainfall | –0.098 | 0.040 | **0.014** |
| **(D)** Inter-birth interval | $\chi^2$ = 5.425 | Intercept | 0.324 | 0.059 | [a] |
| | df = 2 | Age | 0.078 | 0.053 | 0.142 |
| Age and quadratic age | $p$ = 0.066 | $Age^2$ | –0.188 | 0.112 | 0.094 |
| | | N adult females | –0.016 | 0.053 | 0.761 |
| | | Age at first birth | –0.039 | 0.048 | 0.414 |
| Females with known age | | Age at last birth | 0.015 | 0.055 | 0.791 |
| | | Rainfall | –0.111 | 0.060 | 0.063 |

*Significant effect in bold.* [a]*Not shown as has no meaningful interpretation.*

reproduction as well as total number of infants did not differ widely between these groups of females, suggesting that their ages can be reliably estimated and that the age effects found in studies that relied on age estimates (see above) are probably robust.

In wild Verreaux's sifakas, four females were still alive after having given birth for at least an IBI + 2*SD, which is suggestive of reproductive completion (Alberts et al., 2013), but two of them were only about 8 years old, indicating that these long IBIs might be due to other reasons, like poor health or undetected abortions. Only one very old captive Coquerel's sifaka female did not reproduce, and her long IBI is also difficult to interpret because it might have been the result of husbandry decisions. In wild and captive *Eulemur* populations, we found differences in the number of females reaching various ages as well as in the proportion of them giving birth in their respective last year of life, with females in the wild reproducing almost every year until very old age. Also, after having given birth to the last live infant, only five wild redfronted lemur females lived beyond the mean IBI + 2*SD. In captive red lemur females, even 18 females lived longer than the mean IBI + 2*SD, but because of management interventions, these data have to be treated with great caution (see below). In any event, there is evidence to suggest that – as in many other mammals – there is little scope for these lemur females to exhibit grandmaternal care, but we do not know at present whether females live on average longer than males; a pattern found among the few species with grandparental care (Péron et al., 2019). The significant decline in reproductive rates in wild redfronted lemurs contrasts with previous reports for other members of both, the Lemuridae (Gould et al., 2003; Ichino et al., 2015) and Indridae (Richard et al., 2002; Wright et al., 2008), making it difficult at present to derive general conclusions about this aspect of life-history evolution in this basal group of primates.

## Prediction 2: Effects of (Reduced) Adversities

In captivity, animals are buffered from fluctuations and limitations of food availability as well as from adverse environmental factors like droughts, extreme temperatures and parasitism. In addition, veterinary care contributes to improved health, so that overall, more energy should be available for growth and reproduction. As a result, captive females should be able to

**TABLE 6 |** Results of the models in captive red lemurs on the probability to give birth.

| Model | Full-null model comparison | Term | Estimate | SE | *P* |
|---|---|---|---|---|---|
| Probability of giving birth | $\chi^2$ = 14.714 | Intercept | –3.035 | 1.303 | [a] |
| | df = 1 | Age | –3.665 | 1.076 | **0.001** |
| Age | $p$ < 0.001 | Longevity | 0.386 | 0.296 | 0.192 |

*Significant effect in bold.* [a]*Not shown as has no meaningful interpretation.*

sustain higher reproductive rates, which should be reflected by shorter IBIs and attenuated or delayed reproductive senescence. Because the most common cause of environmentally driven mortality in the wild, i.e., predation, is essentially eliminated in captivity, females should on average also live longer than in the wild.

These predictions were only partially supported empirically in our study. In the *Eulemur* species, reproductive senescence occurred independent of the environmental conditions they found themselves in. However, although captive and wild sifakas did not differ in their maximal ages at reproduction or IBIs (**Figure 2**), reproductive senescence occurred only in captive, but not in the wild sifakas. The latter finding is in contrast to findings of reproductive senescence in another wild population of Verreaux's sifakas in Beza Mahafaly (Richard et al., 2002; Alberts et al., 2013; Campos et al., 2022). At Beza Mahafaly, older females were less likely to give birth and they were less likely to close long IBIs by having another offspring; an aspect that we did not explicitly model in our approach. The differences between the two populations might be due to differences in sample size (Beza: 116 females; Kirindy: 38 females, 228 births), but a targeted comparison would be required to test this notion explicitly. These two populations differ, however, in the abundance of the fosa, which is absent at Beza Mahafaly, but common in Kirindy Forest (Kappeler and Fichtel, 2012b). Hence, at Kirindy, more older females, which are likely in a less good condition, might have fallen victim to predation, thereby obscuring potential effects of reproductive senescence. This notion is supported by a study of wild female Edward's sifakas, where the fosa is also abundant, but where no indication of a decline in fertility in dentally senescent (aged > 18 years) females was found (Wright et al., 2008). Thus, variation in predation risk needs to be acknowledged in future demographic studies of wild primates.

Whereas more captive red lemurs lived indeed beyond 20 years than wild redfronted lemurs, their IBIs did not differ, and reproductive rates declined with age in both populations (**Figures 4A,B**). IBIs were longer in the captive population, which might be due to husbandry decisions. In the wild, however, IBIs did not co-vary with female's age. Overall, the IBIs of wild and captive congers in our study nevertheless exhibited generally similar frequency distributions, with the vast majority of females giving birth every year. IBIs may exhibit such limited plasticity because of the photoperiodic control of lemur reproduction (Rasmussen, 1985; Heldstab et al., 2021), which limits ovulations to a narrow time window of 2–3 months, presumably as an adaptation to pronounced seasonality of most lemur natural habitats (Meyers and Wright, 1993).

Data on reproductive performance in captivity have to be treated with caution, however. Hormonal birth control and management decisions have presumably impacted the data on the onset of reproduction and individual fertility. Females that are capable of reproducing and housed with an adult male, but are not recommended to breed, receive contraception, but these decisions are re-evaluated every year. Thus, these data should therefore be treated and interpreted carefully and serve to illustrate the general point about shortcomings and limitations of life-history data from captive animals.

### Effects of Ecology and Sociality

The present study revealed that neither the number of co-resident females nor annual rainfall predicted variation in the probability of giving birth for wild Verreaux's sifakas. Reproductive rates of wild redfronted lemurs were higher in years with more rainfall, but unaffected by the number of co-resident females. There is evidence that lemur reproduction is challenged by resource availability (reviewed in Kappeler and Fichtel, 2015, Kappeler et al., 2022), so that more rain may improve food availability for reproducing females. The different responses by Verreaux's sifakas and redfronted lemurs might be due to the fact that sifaka reproduction at Kirindy is embedded within an 8-month dry season (Kappeler and Fichtel, 2012b) with little phenological tree activity, whereas redfronted lemur lactation coincides with the onset of the production of flowers and young leaves by most trees, which may benefit from more rain. Similarly, birth rates of ring-tailed lemurs, Milne Edward's sifakas and Verreaux's sifakas at Beza Mahafaly also exhibited some reduction in response to low rainfall or droughts (Richard et al., 2000; Gould et al., 2003; Wright et al., 2008).

Competition by other females did not impact reproductive rates in Verreaux's sifakas. However, in the redfronted lemur data set of females with known age, we found a positive effect on the probability of giving birth with an increase in the number of adult females, even though a previous analysis (Kappeler and Fichtel, 2012b) indicated an increase in the intensity of female competition with increasing numbers of resident females. Thus, more data from other species and study sites are required for a more robust assessment, but, overall, the effect of age on female reproduction appears to be more pervasive than those of ecological and social factors, for which this study revealed no consistent effects.

## CONCLUSION

We investigated effects of age, female competition and variation in food availability on female reproductive performance and IBIs in two sympatric wild populations of true lemurs and sifaka and compared these data with captive populations of the same two genera to identify consequences for reproductive senescence. We found some negative effects of age on reproductive performance in three out of four populations. Wild and captive populations did not differ in these life-history traits in the predicted manner, but data from the captive populations were presumably compromised by husbandry decisions. In the wild, only some females live long beyond their last birth, offering no evidence for fertility completion in these basal primates, but reproductive senescence appears to set in shortly before the end of their natural lifespan. Our study contributes additional data on age-related life-history adaptations in monotocous primates and, hence, to a more comprehensive appraisal of reproductive senescence.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found doi: 10.6084/m9.figshare.20036558.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Caff/Core Ministry of the Environment, Madagascar.

## AUTHOR CONTRIBUTIONS

PK and CF collected the data and conceived research. LPr and CF analyzed data. LPe and PK drafted the manuscript. All authors contributed to writing the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.894344/full#supplementary-material

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EDITED BY
Christos Rumbos,
University of Thessaly, Greece

REVIEWED BY
Gabriel Pigeon,
Université de Sherbrooke, Canada
Phyllis Lee,
University of Stirling, United Kingdom

*CORRESPONDENCE
Agathe Crosland
agathe.crosland@u-bourgogne.fr

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# "Born with a silver spoon in the mouth has bad sides too": Experimentally increasing growth rate enhances individual quality but accelerates reproductive senescence in females of the mealworm beetle, *Tenebrio molitor*

Agathe Crosland*, Thierry Rigaud, Aude Balourdet and Yannick Moret

UMR CNRS 6282 BioGéoSciences, Équipe Écologie Évolutive, Université Bourgogne-Franche Comté, Dijon, France

Senescence occurs because of the decline of the strength of selection with age, allowing late-life reduced performances not being counter selected. From there, several phenomena may explain late-life reduced performances, such as the accumulation of deleterious mutations, the expression of pleiotropic genes or the existence of resource trade-offs between early and late performances. This latter phenomenon is at the core of the disposable soma theory of aging, which predicts that growth and early-life reproduction have costs that increase reproductive and actuarial senescence. Whereas the impact of the cost of early reproduction on reproductive and actuarial senescence has been extensively studied, that of the cost of growth remains overlooked and often inconclusive, possibly because of confounding effects associated with the procedures used to manipulate growth rate. Here, we investigated the cost of growth rate and its impact on reproductive senescence and longevity of females of the mealworm beetle, *Tenebrio molitor*. For this purpose, we generated insects with contrasted growth rates by raising groups of them in conditions below, above and optimal relative humidity (RH: 55, 85 and 70%, respectively) during the larval stage. The resulting adult females then bred, under the same optimal RH conditions, early in life, then later in life and were followed there until death. We found that larvae grown under the highest relative humidity exhibited the highest larval growth rate, thanks to both shorter growth duration and the achievement of heavier pupae mass. Adult females from this favorable growing condition lived longer, were more fecund early in life, but suffered from lower late-life reproductive investment. Our study shows that growth rate, which is highly

dependent on the early-life environment, is an important factor modulating adult reproductive senescence, through the occurrence of early-late life trade-offs.

KEYWORDS

aging, cost of growth, disposable soma theory, experimental manipulation, reproductive investment, longevity, holometabolous insect

## Introduction

Senescence (or aging) is a quasi-universal phenomenon in multicellular organisms, defined by the age-related decrease of life-history features related to individual fitness, mainly survival and reproduction. This phenomenon is nevertheless variable both within and among species (Jones et al., 2014; Flatt and Partridge, 2018). Evolution of aging occurs because the strength of age-specific selection, which is maximal during pre-reproductive development, declines after sexual maturation with advancing adult age (Medawar, 1952; Williams, 1957; Hamilton, 1966; Moorad et al., 2019). Although this decrease of selection with age and the supposed accompanying aging is not an universal rule (Baudisch, 2005; Jones et al., 2014; Wensink et al., 2017), it seems accurate for organisms with finite growth, an age structure and well-defined somatic and germline lines (Charlesworth, 2000). In this context, the antagonistic pleiotropy theory of aging proposes that the expression of genes having early-life beneficial effects will be selected even if they cause deleterious effects later in life (Williams, 1957). Such a trade-off between early and late-life performances is also central to the disposable soma theory of aging (Kirkwood, 1977), which may represent a physiological explanation of the antagonistic pleiotropy theory (Lemaître et al., 2015; Maklakov and Chapman, 2019). The disposable soma theory of aging predicts that, as resource demands for growth or reproduction are high, organisms have reduced options to maintain their own body condition, thereby increasing senescence and reducing future fertility and survival through physiological costs incurred. Therefore, individuals should optimize their allocation of resources between growth, reproduction and somatic maintenance according to their average lifespan or the risk of environmental mortality.

A large number of studies have attempted to challenge the predictions of a trade-off between early-life and late-life performances, through laboratory experiments and field longitudinal studies (Partridge et al., 1999; Metcalfe and Monaghan, 2003; Lee et al., 2013; Lemaître et al., 2015). While there is ample evidence suggesting that strong investment in early reproduction is associated with increased rates of reproductive or actuarial senescence (with differences between sexes, e.g., Lemaître et al., 2015; Jehan et al., 2020), evidence of trade-offs between growth and late-life fitness traits is relatively scarce and controversial. Individuals may draw considerable benefits from rapid growth, as reaching adult size in a minimum of time reduces vulnerability to environmental disturbances and predators while facilitating early entry into reproduction (Dmitriew, 2011). Nevertheless, individual growth has been found rarely maximal even when resource availability is unlimited, suggesting that higher growth rates (enabled by either a fast growth or large adult size) is costly (Metcalfe and Monaghan, 2003). To date, interactions between growth processes and senescence remain scarcely explored. For instance, in the three-spined stickleback, fast-growing individuals exhibit shorter longevity, suggesting that enhanced resource allocation to body development early in life increases actuarial senescence (Lee et al., 2013). In the damselfly *Lestes viridis*, experimentally-induced rapid larval development (but not growth rate) reduces adult life span by increasing oxidative damage (Janssens and Stoks, 2018). Other studies failed to detect such early-late life trade-offs. This is the case of a study comparing two ecotypes of the snake, *Thamnophis elegans*, characterized by a contrasted pace of life (fast vs. slow living pace of life) (Sparkman et al., 2007). However, the incomplete knowledge of the environmental conditions experienced early in life by the two ecotypes and the fact that snakes are growing continuously may have prevented the evidence of such a trade-off. In another study, the growth rate of males of the antler fly, *Protopiophila litigata*, was altered by the experimental manipulation of the larval diet, generating variation in development time, which was positively correlated with lifetime mating rate but not with reproductive senescence (Angell et al., 2020). As a result, more research is needed into the early-late trade-off between growth rate and early reproductive success, as well as their respective impacts on late-life reproductive success and senescence. Our study intends to elucidate these relationships by changing growth rate experimentally.

Modifying the amount of food or the quality of the diet provided to juveniles in order to manipulate growth is *a priori* reasonable, as the expression of trade-off is often dependent on resource availability (Metcalfe and Monaghan, 2003; Cohen et al., 2019). Nevertheless, this approach may have several drawbacks when confronted with the phenomenon

of compensatory growth after periods of starvation (e.g., Metcalfe and Monaghan, 2003; Kecko et al., 2017; Ziegelbecker and Sefc, 2021), or when aging does not depend on early diet (Zajitschek et al., 2009), leading to contradictory or inconclusive results. Moreover, increasing growth rate by setting highly favorable conditions may generate individuals with a higher fitness, called the "silver spoon" effect (Grafen, 1988; Lindström, 1999; Monaghan, 2008), which may prevent detection of senescence (Angell et al., 2020). There is therefore a need to explore further the early-late trade-off between growth rate and reproductive senescence using experimental approaches *a priori* avoiding resource alteration during growth to manipulate growth rate. We propose here to study this relationship using a biological model where pre-adult growth can be easily discriminated against (holometabolous insect, where immature stages are strictly different from the sexually mature stage), can be manipulated independently of the diet, and for which experimental conditions are close to those of "field" conditions, to circumvent the problem of field-laboratory differences (Metcalfe and Monaghan, 2003).

This biological model is the mealworm beetle *Tenebrio molitor*. This insect "naturally" lives in stored grain product installations, in which it is considered as a pest. This insect is also attracting considerable interest for its cultivation as a source of food and feed, leading to the development of mass rearing installations (Ribeiro et al., 2018; van Huis, 2021). Living conditions of this insect in the laboratory and "natural" conditions are therefore less different from most other species. Moreover, this insect species seems to fit the preconditions of the disposable soma theory such as finite growth, age structure, and well-separated somatic and germline lines (Charlesworth, 2000). Under the conditions considered to be the most favorable (about 75% RH and 25°C (Punzo and Mutchmor, 1980)), larvae are taken about 17 weeks to reach the pupal stage, this latter stage lasts about ten days (Crosland, personal observations). Adults emerging from pupae can live for several months and do not grow anymore. Individuals are fertile throughout their adult life and this from their fifth day post-eclosion at the earliest. Females exhibit a peak of fertility from day 10 to 20 post-eclosion, which subsequently decreases until death (Dick, 1937; Jehan et al., 2020). To modify the growth rate of *T. molitor*, we adjusted the relative humidity during its larval development. Relative humidity has already been shown to influence growth duration and adult mass (Leclercq, 1948). Humidity might mediate food absorption and metabolism, independently of the amount or the quality of food provided (Hardouin and Mahoux, 2003; Ribeiro et al., 2018). Three relative humidity levels (55, 75, and 85% RH) were used, reported to be non-detrimental to larval survival (Punzo and Mutchmor, 1980).

We first controlled for the success of the modification of larval growth rate by this process: the higher the relative humidity in the larval environment, the faster the larvae developed and the larger the animals were. Females from larvae reared in these contrasted conditions showed differences in survival (longer longevity in adults from larvae grown at 85% RH). The females that had the higher developmental rate were also those that exhibited a higher fecundity early in life, but not late in life. The trade-off between high early developmental rate and reproductive senescence was demonstrated only in the condition where growth rate was higher (exceeding 1 mg/day).

## Materials and methods

### Insect culture and larval environmental conditions

Age-controlled experimental larvae were obtained among the progeny of insects from an outbred stock culture (150 females and 100 males of about 14–21 days post-eclosion) kept together for 3 days in a plastic tank (L 56.5 × l 36.5 × h 15 cm) with bran flour in standard laboratory conditions (24 ± 1°C, 70 ± 5% RH and in permanent darkness). When the larvae had reached 4 weeks old (size from 0.8 to 1.2 cm), 180 of them were isolated and haphazardly distributed into three relative humidity conditions: 55 ± 5%, 70 ± 5%, and 85 ± 5% (designated as larval RH thereafter). For all these three conditions, the temperature was set at 24.5 ± 2°C and the larvae were supplied *ad libitum* with a 9:1 mix of bran flour:fish food (JLB, Novo Malawi). After pupation, all individuals were transferred, still isolated, at 24.5 ± 1°C, 70 ± 5% RH and in permanent darkness. Therefore, only larval development was under different environmental conditions to manipulate growth rates only.

### Growth duration and survival

Isolated larvae were observed twice a week until pupation. Development time (or growth duration) was considered from the egg laying date (estimated as the median date of the 3-day period during which their parents reproduced) to the date of pupation. Pupae were weighed (OHAUS Discovery DV114C balance, $d$ = 0.1 mg) to determine their growth rate, which was calculated as the ratio of pupal mass (in mg) to growth duration (in days) from the egg laying date, when body mass was assumed to be null. Because the larvae were kept in the same environment (same tank) before being haphazardly assigned to their respective RH treatments, they were not supposed to have a distinct body mass. After pupation, all individuals were transferred at 24.5 ± 1°C, 70 ± 5% RH and in permanent darkness. Pupae were observed twice a week until eclosion. New-born adults were weighed and sexed. After eclosion, individuals were maintained in the same environmental condition as were the pupae, supplied with the same bran flour-fish food mix than during the larval stage and provided with a little cube of apple (about 4 mm side) weekly

as a water source. Survival of individuals was checked weekly until the death of all of them. Individual size was estimated after death by measuring the length of both elytra using a Nikon SMZ 1500 stereo microscope with NIS Elements AR software (version 4.00.03). The length of each elytron was measured from its insertion into the scutellum to its basis. All measures were made by the same person with a precision of 0.01 mm. The average value of the two measures per insect was used as a data point.

## Age-specific reproductive performance

Reproduction was analyzed on females only because data on egg production are relevant of their reproductive investment, whereas reproductive investment in males is rather linked to copulation rate, which was not assessed in this study. Reproductive performance of each female beetle was assessed through two reproductive episodes of 10 days: an early one, from day 10 to day 20 post-eclosion, and a late one, from day 40 to day 50 post-eclosion. This early reproductive episode corresponds to the age where females are the most fertile, while the "late" reproduction corresponds to the age period when fertility is declining, but at which mortality is low enough to minimize selective disappearance (Jehan et al., 2020). The reproductive partners (a novel one in each reproductive episode, assigned randomly) were 10-to-20-day old, virgin, males and came from the stock culture. The females were weighed before and after each reproductive episode. During each reproductive episode, a female and her partner were placed in a plastic Petri dish (9 cm diameter) containing a thin layer of bleached flour, a 2 ml tube of water clogged with cotton wool and a piece of apple. To prevent eggs from hatching before being counted, each couple was transferred to a new Petri dish at the middle of the 10-day reproductive period. Eggs were counted after sifting the flour (mesh size: 600 μm). Between the early and the late-life reproductive episodes, and after the late reproductive episode, the experimental individuals were kept isolated, at 24.5 ± 1°C, 70 ± 5% RH and in permanent darkness.

## Statistical analysis

Data on larval development and reproduction were analyzed using R (version 4.1.2). Models presented in the results are those minimizing the Akaike's information criterion (with a $\Delta AIC \leq 2$) and containing the fewest elements in an exhaustive list of models integrating variables and interactions of interest (Galipaud et al., 2017). Larval RH was coded as a factor (three modalities: 55, 70, and 85% RH).

We first tested whether our three experimental larval RH treatments affected female larval growth duration, pupal mass, growth rate, elytra length, survival (as the adult age at death), and total fecundity (calculated as the sum of eggs laid during the two reproductive episodes, R1 + R2). An ANOVA followed by a Tukey's HSD test (functions HSD.test, agricolae package – de Mendiburu, 2021) were computed for larval growth duration, elytra length and total fecundity. For pupal mass and larval growth rate, a Kruskal–Wallis test followed by non-parametric pair comparisons (function kruskal, package agricolae—de Mendiburu, 2021) were computed due to an over-dispersion in the females raised at 85% RH. For survival, a Kaplan–Meier analysis (functions Surv and survdiff, survival package—Therneau et al., 2022) was followed by a pairwise log-rank comparison (function pairwise_survdiff, package survminer—Kassambara et al., 2021). All females that reached pupation were included in the analyses of growth duration, pupal mass and growth rate (55% RH: $n = 32$; 70% RH: $n = 29$; 85% RH: $n = 33$). Three females raised at 70% RH and four raised at 55% RH were not included in the analysis of the elytra length because their elytra were damaged. Only females that had no problem during both reproductive episodes (e.g., death of the partner) were considered for the total fecundity analysis (RH were: 55% RH: $n = 28$; 70% RH: $n = 28$; 85%: $n = 30$).

The effect of larval RH on both female egg production and reproductive investment (as the number of eggs laid per milligram of female, i.e., corrected for the mass of individuals before the reproductive episode considered) was analyzed using linear mixed models (lme function, package nlme—Pinheiro et al., 2022). The most complex models included reproductive episode (early vs. late), larval RH (RH: 55, 70, and 85%), growth rate (mg/day), and life expectancy at the beginning of each reproductive episode as main effects, and the two-way interactions between reproductive episode, larval RH, and growth rate. To adjust for selective disappearance, life expectancy at each reproductive episode was included as an independent covariate (Tarwater and Arcese, 2017). Female identity was included as a random factor on the intercept. After model selection with models computed at maximum likelihood, parameters were estimated by the same model but computed by maximizing the restricted log-likelihood (Zuur et al., 2009). Sample sizes for each larval RH were: 55% RH: $n = 26$; 70% RH: $n = 28$; 85%: $n = 30$. Even if our main hypothesis concerned the influence of growth rate on reproduction, we opted to include larval RH in the model selection despite its collinearity with growth rate. Indeed, larval RH seemed to influence more than just the growth rate. A summary of the best models according to AIC is available in **Supplementary material** (**Supplementary Table 1** for the number of eggs and **Supplementary Table 2** for the reproductive investment).

In addition, links between growth rate (mg/day), early reproduction (R1), late reproduction (R2), and adult longevity were investigated through a path analysis. The hypotheses tested in the model, according to the disposable soma theory, were: (i) individuals with a higher growth rate would be more productive in early reproduction but less in late one, (ii) individuals with

a higher growth rate would live shorter adult life, and (iii) individuals more productive during reproduction would die earlier. From these hypotheses, we constructed four diagrams (see **Supplementary Figure 1** in **Supplementary material**). The significance of relationships between variables and their effects were determined using the package lavaan (function sem) (Rosseel et al., 2022). The best diagram was the one minimizing AIC (with a $\Delta AIC \leq 2$) and minimizing of the number of links. Five model-fit indices were also considered: a chi-square test with as null hypothesis the adequacy of the model to the data, the Comparative Fit Index, the Tucker–Lewis Index, the Root Mean Square Error of Approximation, and the Standardized Root Mean Square Residual (Rosseel et al., 2022).

Our analysis of reproductive senescence relied on a senescence index: the difference in egg numbers between the late reproductive episode and the early one, corrected by the total fecundity of the female. This correction accounting for the total fecundity allows scaling individuals.

$$\text{Reproductive senescence} = \frac{\text{Late number of eggs} - \text{Early number of eggs}}{\text{Late number of eggs} + \text{Early number of eggs}} \quad (1)$$

Reproductive senescence, therefore, occurs if this index is negative. Linear models conducted included larval RH, growth rate, and/or age at death as main effects, and the interaction between larval RH and growth rate. Individual age at death was used as an indicator of individual quality. A summary of the best models according to AIC is available in the **Supplementary material** (**Supplementary Table 3**).

# Results

## Effect of larval environment on growth duration, pupal mass, growth rate and elytra length of females *Tenebrio molitor*

The six traits analyzed, larval growth duration, pupal mass, larval growth rate, adult elytra length, adult survival, and total fecundity, were all significantly affected by the larval RH (growth duration: $F_{2,91} = 102$, $p < 0.0001$; pupal mass: $\chi^2_2 = 51.9$, $p < 0.0001$; growth rate: $\chi^2_2 = 78.3$, $p < 0.0001$; elytra length: $F_{2,88} = 50.6$, $p < 0.0001$; survival: $\chi^2_2 = 17.1$, $p = 0.0002$; total fecundity: $F_{2,83} = 5.11$, $p = 0.008$) (**Figure 1**). The higher the relative humidity was in the larval RH, the less time the larvae needed to reach pupation (**Figure 1A**), and the heavier were the pupae (**Figure 1B**). Because growth rate values were calculated from these two latter measures, they unsurprisingly increase as larval relative humidity increases (**Figure 1C**). Elytra length was similar between females grown at 55 and 70% RH whereas females grown at 85% RH were larger (**Figure 1D**). Adult survival was similar between females reared at 55 and 70% RH (55% RH: 129 ± 9 days; 70% RH: 128 ± 8 days). Females grown at 85% RH lived about 30 days longer than females reared at 55 and 70% RH (85% RH: 159 ± 10 days) (**Figure 1E**). Females reared at 85% RH produced more eggs during the two reproductive episodes cumulated than females grown at lower relative humidity (55% RH: 126 ± 6 eggs; 70% RH: 129 ± 6 eggs; 85% RH: 148 ± 5 eggs) (**Figure 1F**).

## Reproductive performance

Females laid significantly more eggs during their early reproductive episode than during the late one (76.0 ± 2.4 eggs vs. 58.8 ± 1.9 eggs, respectively) (**Table 1** and **Figure 2A**). In general, the higher the female growth rates during larval stages, the higher the egg production (**Table 1** and **Figure 2A**). However, this increase in female fecundity was contrasted according to the reproductive episodes considered (**Table 1** and **Figure 2A**). Indeed, the positive association between egg production and growth rate was only significant during the early reproductive episode ($F = 12.68$; d.f. = 1 on 82; $p < 0.001$) but not during the late one ($F = 0.12$; d.f. = 1 on 82; $p = 0.735$) (**Figure 2A**). In addition, female life expectancy was positively associated with fecundity (**Table 1**): the most fecund females in the two reproductive episodes measured here were also those that lived the longest (**Figure 2B**).

Female's reproductive investment (number of eggs per mg of weight) was significantly higher during the early reproduction episode than during the late one (0.64 ± 0.02 eggs/mg vs. 0.53 ± 0.02 eggs/mg, respectively) (**Table 2** and **Figure 3A**). Growth rate also influenced reproductive investment: the higher the growth rate, the lower the reproductive investment (**Table 2** and **Figure 3B**). Females that lived the longest after reproduction were those that invested the most in reproduction according to their body mass (**Table 2** and **Figure 3C**).

## Causality network between growth rate, early/late fecundity and adult longevity

As shown above in the analysis of reproductive performances, the path analysis revealed that females that grew faster laid more eggs during the early reproductive episode and lived longer (**Figure 4**). The link between early and late fecundity was not significant. Finally, the more fecund the females were late in their life, the longer they lived (**Figure 4**).

## Reproductive senescence

The reproductive senescence index was significantly affected by the interaction between growth rate and larval RH (**Table 3**). There was no significant relationship between

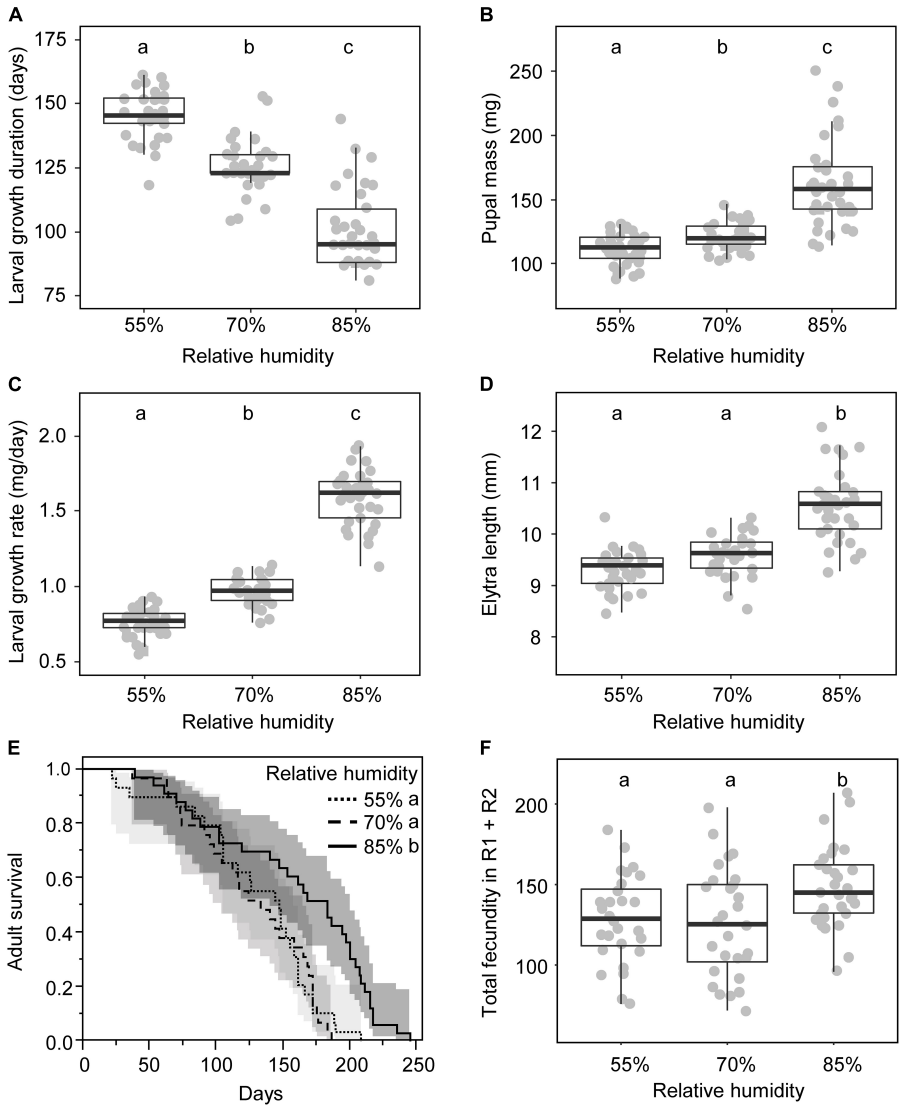


FIGURE 1
Variation of life-history traits according to conditions of larval growth (relative humidity): growth duration **(A)**, pupal mass **(B)**, growth rate **(C)**, elytra length **(D)**, adult survival **(E)**, and total fecundity (sum of the number of eggs laid during the early and the late reproductive episode) **(F)**. Different letters indicate significant differences assessed by post-hoc tests [Tukey HSD in panels **(A,D,F)**, Kruskal–Wallis with Bonferroni correction in panels **(B,C)** and Log-rank pairwise tests in panel **(E)**]. In panels **(A–F)**, gray dots are observations; box plots are median (bold lines), interquartile (boxes) and interdecile (error bars). In panel **(E)**, confidence interval curves are given for 95%.

TABLE 1 **Linear mixed model analyzing the number of eggs laid by *Tenebrio molitor* females.**

| Fixed variables | Effect | 95%CI | d.f. | *F* | *p* |
|---|---|---|---|---|---|
| Reproductive episode | | | | | |
| Late | 7.63 | (−10.01; 25.26) | 1, 81 | 39.54 | <0.0001 |
| Growth rate | 19.76 | (8.40; 31.13) | 1, 82 | 7.89 | 0.006 |
| Life expectancy at reproductive episode | 0.08 | (0.01; 0.14) | 1, 81 | 5.00 | 0.028 |
| Reproductive episode : Growth rate | | | 1, 81 | 7.39 | 0.008 |
| Late | −20.20 | (−34.99; −5.41) | | | |

The model presented here is the only one minimizing the AIC with a $\Delta$AIC $\leq$ 2 (see Supplementary Table 1 in Supplementary material). Sample size: 55% RH: $n$ = 26; 70% RH: $n$ = 28; 85%: $n$ = 30. Standard deviation of Individuals' random intercept: 6.79, 95%CI (2.88; 16.02), $R^2$ = 0.35. Effects involving reproductive episodes are given for the late episode compared to the early one (reference level).

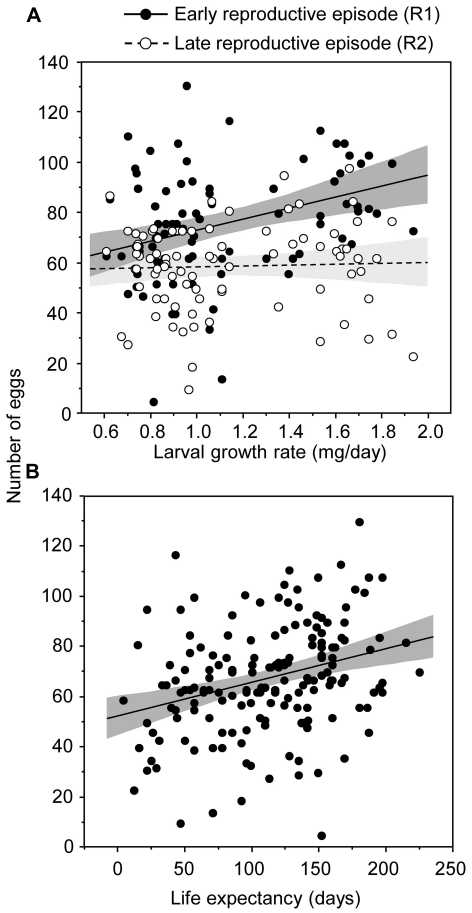


FIGURE 2
Number of eggs laid by *Tenebrio molitor* as a function of the female's growth rate according to the reproductive episode (early reproductive episode, R1: females 10–20 days old post-eclosion; late reproductive episode, R2: females 40–50 days old post-eclosion) **(A)** and as a function of life expectancy at reproductive episode **(B)**. Each dot represents the measure of a single female during one reproductive episode. Lines are linear regressions with 95% confidence intervals.

TABLE 2 Linear mixed model analyzing the reproductive investment (number of eggs per mg of body mass before the reproduction episode) of *Tenebrio molitor* females.

| Fixed variables | Effect | 95%CI | d.f. | *F* | *p* |
|---|---|---|---|---|---|
| Reproductive episode | | | 1, 82 | 22.21 | <0.001 |
| Late | −0.089 | (−0.138; −0.04) | | | |
| Larval RH | | | 2, 80 | 2.32 | 0.105 |
| 70%RH | 0.162 | (0.031; 0.293) | | | |
| 85%RH | 0.052 | (−0.007; 0.11) | | | |
| Growth rate | −0.39 | (−0.602; −0.178) | 1, 80 | 14.89 | <0.001 |
| Life expectancy at reproductive episode | 0.001 | (0; 0.001) | 1, 82 | 6.03 | 0.016 |

Model presented here are the ones minimizing the AIC with a $\Delta$AIC $\leq 2$ and integrating the least number of variables (see **Supplementary Table 1** in the **Supplementary material** for other best models). Sample size: 55% RH: $n = 26$; 70% RH: $n = 28$; 85%: $n = 30$. Standard deviation of Individuals' random intercept: 0.06, 95%CI (0.03; 0.13) $R^2 = 0.33$. Effects involving reproductive episodes are given for the late episode compared to the early one (reference level). Effects involving the larval RH are given compared to 55%RH (reference level).

growth rate and reproductive senescence in females that grew at 55% ($F = 0.65$; df = 1; $p = 0.430$) and 70% RH ($F = 3.11$; df = 1; $p = 0.090$) (**Figure 5**). However, females from larva kept at 85% RH showed stronger reproductive senescence when having grown faster ($F = 9.60$; d.f. = 1; $p = 0.004$) (**Figure 5**).

# Discussion

## Higher relative humidity increases larval growth rate in *Tenebrio molitor*

By manipulating the relative humidity during the larval stages of *T. molitor*, we successfully manipulated the growth rate. The larvae reared at higher relative humidity developed faster. This enhanced growth rate resulted from both a shorter growth duration and an increase in body mass when reaching the pupal stage. Such a result was expected, as the relative humidity is known to modify *T. molitor* larval development (Leclercq, 1948). A mechanism proposed but not tested yet to our knowledge, is that higher relative humidity might enhance the capacity to absorb water and so to ingest food, increasing metabolism, irrespectively of the food quality and quantity provided (Hardouin and Mahoux, 2003; Ribeiro et al., 2018). Indeed, although *T. molitor* is an insect living on dry substrates and is efficient at extracting and saving water, its optimal relative humidity is around 70% (Ribeiro et al., 2018 for a review). Such an effect of relative humidity on body mass might be a potential source of confounding effect when attempting to understand how growth rate is trading-off against future performances and especially future reproduction (Metcalfe and Monaghan, 2003). It is widely known that the body mass of animals, and especially insects, is directly linked with individual body condition and often predicts fecundity (Arrese and Soulages, 2010; Zanchi et al., 2019). However, it seems illusory to think that changing larval growth rate can be done without affecting adult size in *T. molitor*. We have controlled for this effect of body size in our statistical analyses, but it never directly affected female fertility nor their reproductive investment (see below). Additional experimental methods enabling to generate variation in larval growth rate might provide complementary information regarding early-late life trade-offs, at least to figure out whether they all converge toward the same results. They may involve the modification of the larval environment by other means than

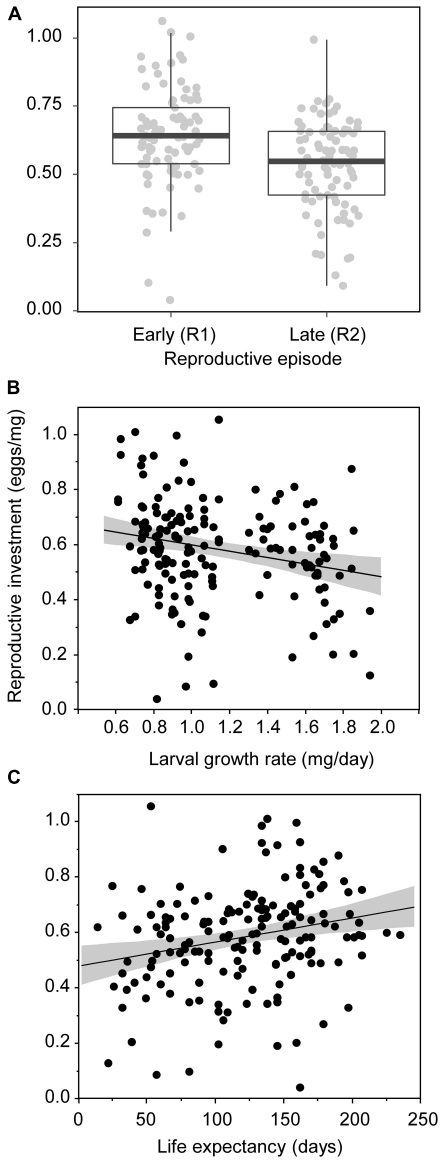


FIGURE 3
Reproductive investment (number of eggs per mg of body mass) of females *Tenebrio molitor*, according to reproductive episode (early reproductive episode, R1: females 10–20 days old post-eclosion; late reproductive episode, R2: females 40–50 days old post-eclosion) **(A)**; as a function of the female's growth rate **(B)** and as a function of life expectancy at reproductive episode **(C)**. Each dot represents the measure of a single female during one reproductive episode. Lines are linear regressions with 95% confidence intervals.

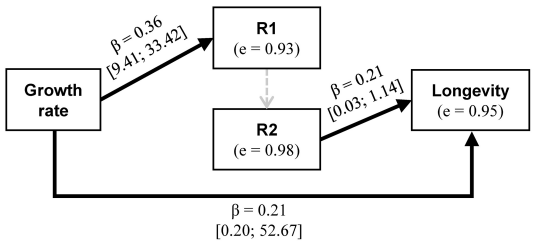


FIGURE 4
Path analysis diagram describing the direct and indirect effects of growth rate on fecundity and adult longevity (best one of four tested; see **Supplementary Figure 1** in **Supplementary material** for other diagrams). Arrows with solid black lines represent significant ($p$-value <0.05) links, arrows with dashed lines represent links tested but insignificant. When the relationship was significant, the standardized coefficient (β) and 95% confidence interval of the non-standardized coefficient are given. Errors (e) are given for each endogenous variable as $\sqrt{(1-R^2)}$.

the manipulation of the relative humidity, such as manipulating food or temperature. They may also involve the use of different strains of *T. molitor* differing in their larval growth rate (e.g., inbred lines or lines obtained after from selection experiments). At least, our present study succeeded in generating individuals that have experienced different growth conditions during the larval stages only. The adults were all kept in a common garden. Thus, if, as suspected, changes in larval growth rate between the RH treatment modalities might be caused by metabolic changes, they were due to differences in the larval environment only.

## Does increasing growth rate of *Tenebrio molitor* induce a "silver spoon" effect?

The experimental modification of the environmental relative humidity of larvae affected the survival of *T. molitor* adult females. There were no differences between individuals reared at 55 and 70% RH, but the ones reared at 85% RH survived longer. The same tendency was observed for the total fecundity. In addition, the longer the females are living after the last reproductive episode, the more eggs they have laid and the more they have invested in reproduction. This observation is congruent with the absence of terminal investment and rather militates for the hypothesis of the use of a reproductive restraint strategy in this insect species, as previously proposed (Jehan et al., 2021, 2020). The higher reproductive performances of long-lived females may also be linked to their individual quality. Indeed, considering the fact that females reared at 85% RH during their larval stages were also those that lived the longest, were the largest, and laid more eggs (see below), they may benefit from a "silver spoon" effect, where individuals born in good conditions have fitness advantages later in life (Grafen, 1988; Lindström, 1999; Monaghan, 2008). Such an effect has already been observed—not consistent with all life-history traits, however—in an experimental study manipulating larval diet in an insect (Angell et al., 2020). Alternatively, high environmental relative humidity may represent cues for a harsher environment,

TABLE 3 Linear model analyzing the reproductive senescence of *Tenebrio molitor* females.

| Variables | Effect | 95%CI | d.f. | *F* | *p* |
|---|---|---|---|---|---|
| Larval RH | | | 2 | 2.74 | 0.07 |
| 70%RH | 0.74 | (−0.05; 1.52) | | | |
| 85%RH | 1.00 | (0.09; 1.91) | | | |
| Growth rate | 0.33 | (−0.20; 0.85) | 1 | 1.09 | 0.30 |
| Larval RH: Growth rate | | | 2 | 4.63 | 0.013 |
| 70%RH | 0.70 | (−1.56; 0.16) | | | |
| 85%RH | −0.81 | (−1.76; 0.13) | | | |
| Residuals | | | 80 | | |

The reproductive senescence was estimated as the ratio of the difference between the two reproductive episodes (early and late) over the total of eggs laid during both reproductive episodes. The models presented here are the ones minimizing the AIC with a $\Delta AIC \leq 2$ and including the least number of variables (see **Supplementary Table 3** in **Supplementary material**). Sample size: 55% RH: $n = 26$; 70% RH: $n = 28$; 85%: $n = 30$, $R^2 = 0.17$.

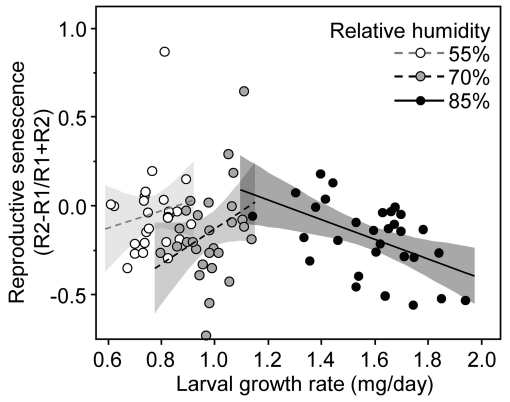


FIGURE 5
Reproductive senescence of females of *Tenebrio molitor* as a function of larval growth rate and according to the conditions of larval growth (relative humidity). Reproductive senescence was estimated by the difference between the late number of eggs (R2) and the early number of eggs (R1) corrected by the total number of eggs during the two reproductive episodes (R1 + R2).

as it could be associated to better conditions for microbial development and therefore higher risk of infection. As a result, females might have adaptively accelerated their reproductive effort (Nettle et al., 2013). However, such an acceleration of reproduction is expected to negatively affect somatic defenses and reduce longevity (Williams, 1957; Kirkwood, 1977; Lemaître et al., 2015; Maklakov and Chapman, 2019). Since our controlled laboratory conditions prevented the development of microbial pathogens and provided *ad libitum* food, the survival cost of accelerated early reproduction could not be revealed. Further study may need to look at the immunity of the insects in such a condition.

## Higher larval growth rate increased reproductive senescence

As expected, the female reproductive performance, in terms of both number of eggs laid and reproductive investment (number of eggs controlled by female body mass), were lower late in life than earlier (Dick, 1937; Jehan et al., 2021). Females that exhibited the highest growth rate laid more eggs during the early reproduction episode, but the larval growth rate did not affect the number of eggs produced during the late one. The index of reproductive senescence we have used, calculated as the difference between the number of eggs laid during the late and the early reproductive episode divided by the total number of eggs laid, provides complementary information. This index has the advantage of taking into account female total fecundity (during both reproductive episodes) and so reduces the influence of individual female quality. According to this index, reproductive senescence was only detected in females reared at 85% RH, namely the fastest-developing ones (because they had the shorter growth duration and the highest pupal mass), exhibiting growth rate values above one mg per day. Our experimental approach did not allowed separating the effect of the onset of senescence and the rate of senescence. Therefore, we cannot tell whether females that developed faster were those that had the earliest of senescence. Nevertheless, our results clearly illustrate the occurrence of pace of life along a slow-fast continuum. Fast-developing females were larger, exhibited higher fecundity early in life, but suffer from steep reproductive senescence later while growing in age. By contrast, slow-developing females were smaller, died earlier, but exhibited stable reproductive performances throughout their lives (at least between the 10 and 50 days of adult life we have measured here). The results of our work are in line with those of two previous studies on insects. Hunt et al. (2004) showed that high-quality cricket males were those that invested the most in reproduction at the expense of reduced longevity. Hooper et al. (2017) showed that high-condition males of a neriid fly were those that had the quickest development, but the fastest reproductive and actuarial senescence compared to low condition males. Since we did not measure the total (lifetime) reproductive success of the females, we cannot exclude that the observed low rate of reproductive senescence among slow-developing females is due to an incomplete reproductive census. However, our protocol, where "only" two reproductive episodes were analyzed—one early in life, when fecundity was predicted to be the highest, and one later in life (from the age of 40 day old onward), when fecundity is known to begin its decline (Jehan et al., 2020)—has the advantage of measuring reproductive senescence before the onset of actuarial senescence. Perhaps recording additional reproductive episodes later in life would allow a comparison of senescence trajectories among females

with contrasted growth rates, but such a study would require disentangling senescence from selective disappearance (Vaupel et al., 1979; Nussey et al., 2011), since mortality is rapidly increasing after 60 days of adult life.

## Conclusion

By manipulating the relative humidity of the environment of the larvae of the mealworm beetle, *T. molitor*, we were able to generate variation in growth rate and adult female quality consistent with a "silver spoon" effect (Grafen, 1988). Despite this effect, and in line with the disposable soma theory of aging, we provide unambiguous evidence that growth rate significantly correlates with reproductive senescence. Overall, our study shows that growth rate, which is highly dependent on the early-life environment, is an important factor contributing to adult life history and senescence through the occurrence of early-late life trade-offs.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.915054/full#supplementary-material

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EDITED BY
Jean-Michel Gaillard,
Université Claude Bernard Lyon 1, France

REVIEWED BY
Marco Festa-Bianchet,
Université de Sherbrooke, Canada
Anthony Michael Pagano,
United States Geological Survey (USGS), United States

*CORRESPONDENCE
Marwan Naciri
marwan.naciri@cefe.cnrs.fr

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# Reproductive senescence in polar bears in a variable environment

Marwan Naciri[1]*, Jon Aars[2], Marie-Anne Blanchet[2], Olivier Gimenez[1] and Sarah Cubaynes[3]

[1]CEFE, Univ. Montpellier, CNRS, EPHE, IRD, Montpellier, France, [2]Norwegian Polar Institute, FRAM Centre, Tromsø, Norway, [3]CEFE, Univ. Montpellier, CNRS, EPHE-PSL University, IRD, Montpellier, France

Reproductive senescence is ubiquitous in mammals. However, patterns of senescence vary across reproductive traits, even within populations, perhaps because of differences in selection pressures, physiological constraints, and responses to environmental conditions. We investigated reproductive senescence in wild female polar bears (*Ursus maritimus*), using 31 years of capture-recapture data from the Svalbard area. We studied the influence of environmental conditions on age-specific litter production and litter size using generalized linear mixed models. Further, using a capture-recapture model that handles the dependency between vital rates of individuals belonging to the same family unit, we assessed maternal-age-related changes in first year cub and litter survival. We provide clear evidence for reproductive senescence in female polar bears. Litter production and litter size peaked in middle-aged females and declined sharply afterward. By contrast cub and litter survival did not decline after prime age. We found no evidence of terminal investment. The reproductive output of all females was affected by sea-ice conditions during the previous year and the Arctic Oscillation, with some effects differing greatly between age groups. Old females were affected the most by environmental conditions. Our results suggest that the decline in reproductive output is a combination of fertility and body-condition senescence, with a weak contribution of maternal-effect senescence, possibly due to benefits of experience. Further, as predicted by evolutionary theory, senescence appears to be a consequence of failures in early stages of the reproductive cycle rather than in late stages, and environmental variation affected old females more than prime-aged females. Our study emphasizes the need to study several reproductive traits and account for environmental variation when investigating reproductive senescence. Differences in senescence patterns across reproductive traits should be interpreted in light of evolutionary theory and while considering underlying physiological drivers.

KEYWORDS

**polar bears, sea ice, fertility, reproductive senescence, litter size, offspring survival, environment, litter production**

## Introduction

Reproductive senescence, the decline in reproductive success with increasing age, is nearly ubiquitous in wild populations of vertebrates (Lemaître and Gaillard, 2017; Lemaître et al., 2020). However, the age at onset and the rate of senescence vary considerably across species (Jones et al., 2014; Lemaître et al., 2020) and populations (Lemaître and Gaillard, 2017). Furthermore, various indicators of reproductive performance, such as conception rate, fecundity, litter size and offspring survival often display contrasted and asynchronous patterns of senescence within populations (Nussey et al., 2009; Massot et al., 2011; Hayward et al., 2013, 2015; Berger et al., 2015). These reproductive traits are likely governed by distinct physiological mechanisms and may be under different selection pressures. Therefore, they may vary differently with age and in response to environmental conditions, potentially explaining the heterogenous patterns of senescence observed across traits and/or populations within the same species. Considering reproductive senescence as the result of senescence of several processes which vary in their physiological drivers and in the timing at which they intervene in the reproductive cycle, while taking into account the influence of environmental conditions, may help gain a better understanding of senescence patterns.

First, reproductive senescence can be decomposed into fertility senescence and maternal-effect senescence (Moorad and Nussey, 2016; Karniski et al., 2018). Fertility senescence corresponds to the aging of the reproductive physiology resulting in lower fertility. A major mechanism underpinning fertility senescence is the progressive depletion of the finite pool of primary oocytes in mammals and birds, resulting in lower pregnancy rates (Lemaître and Gaillard, 2017). Maternal-effect senescence, on the other hand, corresponds to a declining in the ability to provide for offspring (both pre- and postnatally) with increasing age, resulting in lower reproductive success in late-life. Maternal-effect senescence is caused by somatic senescence impairing functions (e.g., resource acquisition, lactation, immunity) that have an influence on offspring traits such as body mass and viability (Karniski et al., 2018). For instance, reduced predatory performance at old age as documented in wolves (MacNulty et al., 2009) may lead to lower energy intake which may hinder lactation, resulting in low body mass of offspring born to old females. In capital breeders, senescence in body condition, a consequence of somatic senescence, may be an additional cause of reproductive decline as it may prevent old females from undertaking reproduction due to insufficient energy stores (Derocher et al., 1992; Nussey et al., 2011).

Second, reproduction can be considered as a sequence of stages, any of which may fail in a given breeding attempt, with reproductive senescence increasing the overall chances of failure. Late stages of the reproductive cycle (potentially mediating maternal-effect senescence, e.g., lactation) contribute more to the fitness cost of overall reproduction (that is, reduced survival and future reproduction) than do earlier, typically less energy demanding, stages (potentially mediating fertility or maternal-effect senescence, e.g., pregnancy) (Clutton-Brock et al., 1989). Losing offspring in early stages of the reproductive cycle should therefore be less costly than losing offspring in late stages. In addition, in species providing parental care over several breeding season, losing offspring tardively entails lost breeding opportunities. Therefore, senescence of traits impacting late stages of the reproductive cycle should be counter-selected more strongly than traits impacting earlier stages of the reproductive cycle (Lemaître and Gaillard, 2017) [but see Nussey et al. (2009)].

The way and extent to which environmental conditions interact with reproductive senescence–through either of its components or any of its stages, also remains poorly understood (Lemaître and Gaillard, 2017; Gaillard and Lemaître, 2020). Early life is the most critical period during which the available resources must be partitioned between somatic maintenance, growth, and first reproductive events. Good environmental conditions during early life (e.g., high resource availability) can have positive impacts later in life, including at old age (Cooper and Kruuk, 2018). Such "silver-spoon" effects have been shown in mammals, e.g., red deer born during a year with high intra-specific competition exhibited higher rates of reproductive senescence (Nussey et al., 2007). Less is known about the effect of environmental conditions faced during adult life (Lemaître and Gaillard, 2017). Based on resource acquisition and allocation theory, age-related differences in reproductive performance are expected to be minimal under favorable environmental conditions and maximum under adverse environmental conditions. These predictions have mostly been tested in seabirds (Ratcliffe et al., 1998; Bunce et al., 2005; Nevoux et al., 2007; Vieyra et al., 2009; Pardo et al., 2013; Oro et al., 2014), showing contrasted results. In Australasian gannets (Bunce et al., 2005) and black-browed albatrosses (Pardo et al., 2013) results were consistent with theoretical predictions. By contrast, differences in breeding performance were highest during years of high food availability in Audouin's gulls (Oro et al., 2014), and only detected in years of intermediate environmental conditions in great skuas (Ratcliffe et al., 1998). Heterogeneity among individuals unaccounted for may explain these differences (Nussey et al., 2008; Gimenez et al., 2018). In mammals, one study on lemurs showed that in years of low rainfall during the lactation period, old females had reduced offspring survival but not younger females, because of tooth wear (King et al., 2005). Similarly, weaning success of young and old female chamois was reduced in poor years while that of prime-aged females remained unchanged (Morin et al., 2016). These findings are in line with theoretical predictions. To our knowledge, the interactive effect of maternal age and environmental variation on reproductive output has not been studied in other mammal species.

Here we investigate age-related patterns of four indicators of reproductive success (litter production, litter size at capture, and cub and litter survival during the first year) in interaction with environmental conditions in female polar bears (*Ursus maritimus*) in Svalbard, Norwegian Arctic, using 31 years (1992–2019, 2021–2022) of capture-recapture (CR) data. Our aim is twofold. (i) We analyze and contrast age-related changes in several indicators of reproductive success to get insight into the contribution of fertility senescence and maternal senescence to overall reproductive senescence. Cub and litter survival fall within the scope of maternal-effect senescence, whereas litter production and litter size may reflect body-condition, fertility and maternal effect senescence (Derocher et al., 1992). (ii) Evaluate the age-specific impacts of environmental variation on our first two indicators of reproductive success to understand the role of environmental conditions in shaping senescence patterns in the wild. Polar bears live in a challenging habitat, where availability of food resources varies greatly seasonally and annually, due to the dynamic nature of sea-ice habitat. These challenges have been aggravated by climate warming, which has progressively led to earlier sea-ice break-up in spring, later freeze-up in autumn, lower extent, and altered sea-ice characteristics. These changes in sea-ice reduce foraging opportunities for polar bears in several areas (Stirling and Derocher, 2012 and Derocher, 2012), with cascading effects on body condition and reproduction (Obbard et al., 2006, 2016; Stirling and Derocher, 2012). Additionally, contrary to other well-studied polar bear populations, Svalbard polar bears are not hunted, following a ban in the 1970'. This context provides us with an ideal setting to investigate how age and a broad range of environmental conditions influence reproductive output.

Female polar bears are capital breeders for the first part of their reproductive cycle. They must acquire extensive fat reserves before entering their den, where they give birth and nurse their young while fasting (Atkinson and Ramsay, 1995). Given the high cost of reproduction, we expect body-condition senescence and maternal-effect senescence to play a major role in polar bears, with strong declines in litter production, litter size, and cub and litter survival in late life. Alternatively, since late stages of the reproductive cycle should be under stronger selection than early stages (Lemaître and Gaillard, 2017), we may observe that cub and litter survival display slower senescence than litter production and litter size. Regarding the effect of environmental conditions, we predict an influence of sea-ice dynamics and the Artic Oscillation index in the year prior to capture, during which pregnant females acquire fat reserves for maternity denning, on reproductive performance. We expect lower reproductive performance for all females following years with a lack of sea-ice, although this effect could be amplified for certain age groups (Gaillard and Yoccoz, 2003). Young females and senescent females could be affected the most (e.g., Morin et al., 2016). Alternatively, additional experience acquired with age may ease the effect of environmental conditions on old females through better foraging ability [e.g., better knowledge of resource distribution, higher hunting success (Daunt et al., 2007; Nisbet et al., 2020)] or higher quality of maternal care (Weladji et al., 2006; Limmer and Becker, 2009).

Overall, our long-term data provides us with an opportunity to study senescence patterns in several indices of reproductive success in a long-lived capital breeder species, under a wide range of environmental conditions.

# Materials and methods

## Study species and data

### Biology of polar bears

The polar bear is a long-lived species, with a maximum life span slightly above 30 in the wild (Weigl, 2005). Polar bears mate in spring, but females delay implantation until autumn (Lønø, 1970; Ramsay and Stirling, 1988). If females' energy stores are too low at this time, they may abort (Atkinson and Ramsay, 1995). Otherwise, they enter a den where they give birth between November and January, most often to two cubs, occasionally to one, or rarely to triplets (Stirling, 2011). Cubs are very small at birth, weighing around 600 g. In the Barents Sea area, females emerge from the den in March–April when their cubs are 3–4 months old (Wiig, 1998). Between den entry and den emergence, females fast, loosing on average 40% of their body mass, which means they must have extensive fat stores at den entry to sustain lactation and maintenance (Atkinson and Ramsay, 1995). For a few days or occasionally weeks after den emergence, families remain close to their den (Hansson and Thomassen, 1983). During their first year, cubs have a high mortality rate (Amstrup and Durner, 1995; Derocher and Stirling, 1996; Cubaynes et al., 2021) and depend entirely on their mother. Singleton litters may result from pre-natal or neonatal mortality occurring in what was initially a twin (or more rarely a triplet) litter. Cubs remain with their mother, depending on her for food and protection, until age 2–2.5, with most departures occurring in late winter and spring (Amstrup, 2003). In Svalbard, most females have their first litter at age 6, although some females can have their first litter at age 5 (Derocher, 2005). Polar bears depend strongly on sea-ice for hunting seals, their main prey (Stirling and Archibald, 1977), for traveling, and for access to denning areas (Derocher et al., 2011). Contrary to other well-studied populations, Svalbard polar bears are not subjected to indigenous subsistence harvest or sport hunting. Since the ban on polar bear hunting in 1973, an average of 2.9 bears are killed by humans each year, mainly because they pose a danger to life or property.[1]

1 https://www.mosj.no/en/influence/hunting-trapping/polar-bear-bag.html

### Longitudinal data on polar bears

We live-captured and marked polar bears around Svalbard (Norway, **Figure 1**) in 1992–2019 and 2021–2022 using methods described in Stirling et al. (1989). Captures occurred between late March and early May when most females with cubs-of-the-year (hereafter "cubs"), have emerged from maternity dens (Wiig, 1998). Age of cubs, yearlings and 2-year-olds was determined with certainty based on size. Age of bears captured for the first time as sub-adults or adults was estimated using an extracted vestigial premolar tooth (Calvert and Ramsay, 1998; Christensen-Dalsgaard et al., 2010). Mother's straight body length (cm) (hereafter "size"), was measured as the straight-line distance between the tip of the nose and the caudal end of the tail bone of bears laying in sternal recumbency (Derocher, 2005). For mothers with cubs, litter size was recorded at capture. Females captured alone were considered to have no dependent offspring alive. This could be so if (i) they did not mate, (ii) they mated but did not get pregnant or aborted, (iii) they lost their cub, yearling, or 2-year-old offspring prior to capture, or (iv) they parted from their 2-year-old offspring prior to capture. Apart from (iv), these situations all correspond to a reproductive failure.

In the 1990s, most of the sampling was conducted using a base station located on Hopen Island (in the south-east of the Svalbard archipelago, **Figure 1**), an important denning area at that time (Derocher et al., 2011). Many females with cubs of the year were thus captured close to the base station just after they had left their maternity den. From 2000 onward, a much larger area of Svalbard was covered, using as a base station either a boat with a helicopter deck or a cabin located in Spitsbergen. We thus think that the probability of capturing females with cubs was higher in 1992–1999 than from 2000 onward. Consequently, in the analysis including both lone females and females with cubs, we only considered captures that occurred in year 2000 or later.

## Environmental conditions

### Sea-ice

We downloaded daily sea-ice concentration grids made available by the university of Hamburg for year 1992–2019 (Kern et al., 2020). These grids are derived from radiance temperature data measured remotely using the 85 GHz Special Sensor Microwave/Imagers (SSM/Is) and Special Sensor Microwave Imager/sounder (SSMIS) channels and processed using the ARTIST Sea Ice algorithm (Kern et al., 2020). The data has a resolution of 12.5 × 12.5 km. Nonetheless, as the Hamburg University timeseries does not extend further back than December 1991, we retrieved daily sea-ice concentration grids (with a 25 × 25 km gird cell size) for 1990 and 1991 from the National Snow and Ice Data Center (Cavalieri et al., 1996).

To define the study area, over which sea-ice covariates were calculated, we used telemetry data from 135 adult female polar bears captured between 1989 through 2021 and fitted with a satellite-linked collar. Polar bear locations were obtained using Argos (CLS, Toulouse, France) or Global Positioning System (GPS) systems. Location estimates became more accurate over the years with a gradual transition from using mainly Argos location systems to GPS with the first GPS deployments occurring in 2000. To account for the larger spatial errors of the Argos system, all Argos locations were processed through a speed, distance, and angle filter (Freitas et al., 2008) which removes all positions deemed unrealistic given a maximum speed and a maximum deviation from the track. In addition, the sampling regime varied between the tags from one position every 2 h to one position every 6 day. Hence, we retained one GPS position every 6th day to match the early Argos sampling regime and not introduce a bias. The tracking duration was at least 1 year. The study area was then defined as the 95% minimum convex polygon (MCP) (Mohr, 1947). This method was selected because it is simple, non-parametric and creates the unique smallest polygon encompassing a preset proportion of locations (here 95%, **Figure 1**).

Following (Cherry et al., 2013; Molnár et al., 2020), we considered a grid cell to be ice-covered if sea-ice concentration >30%. We then generated a 1990–2021 time series at daily resolution of the surface covered by sea-ice (hereafter "sea-ice extent") by multiplying the number of ice-covered grid cells in our study area by the appropriate area of an individual grid cell ($25^2$ and $12.5^2$ $km^2$ for years 1990–1991 and 1992–2021, respectively). We adopted an extent-based rather than concentration-based approach following the recommendations of Molnár et al. (2020). Missing and aberrant values were interpolated using the closest available previous and following days. We defined the transition sea-ice extent as the extent halfway between the 30-day minimum (September) and maximum (March) extent over 1990–1999. For each year, the date of sea-ice break-up (respectively of freeze-up) was defined as the date when sea-ice extent falls below (respectively rises above) the transition sea-ice extent for ≥5 consecutive days. Considering that cells were ice-covered if sea-ice concentration >15% (instead of >30%) as done in Galicia et al. (2020) and Rode et al. (2021) yielded very similar dates of sea-ice break-up (mean difference −0.2 ± 0.04 days, Kendall's $\tau = 0.98$) and freeze-up (mean difference −0.6 ± 0.2 days, Kendall's $\tau = 0.96$).

We expected early break-up and late freeze-up to reduce foraging opportunities and fat storage before denning, as well as access to denning areas, thereby negatively affecting reproductive performance in the following year (Derocher et al., 2011; Molnár et al., 2011; Stirling and Derocher, 2012). We thus considered the date of sea-ice break-up (*DateBreakUp*) and freeze-up (*DateFreezeUp*) in the year prior to capture as covariates.

### Arctic oscillation

The Arctic oscillation (AO) is a large-scale climate index calculated based on the sea-level pressure anomalies north of 20°N. The AO determines the wind regimes around the Arctic

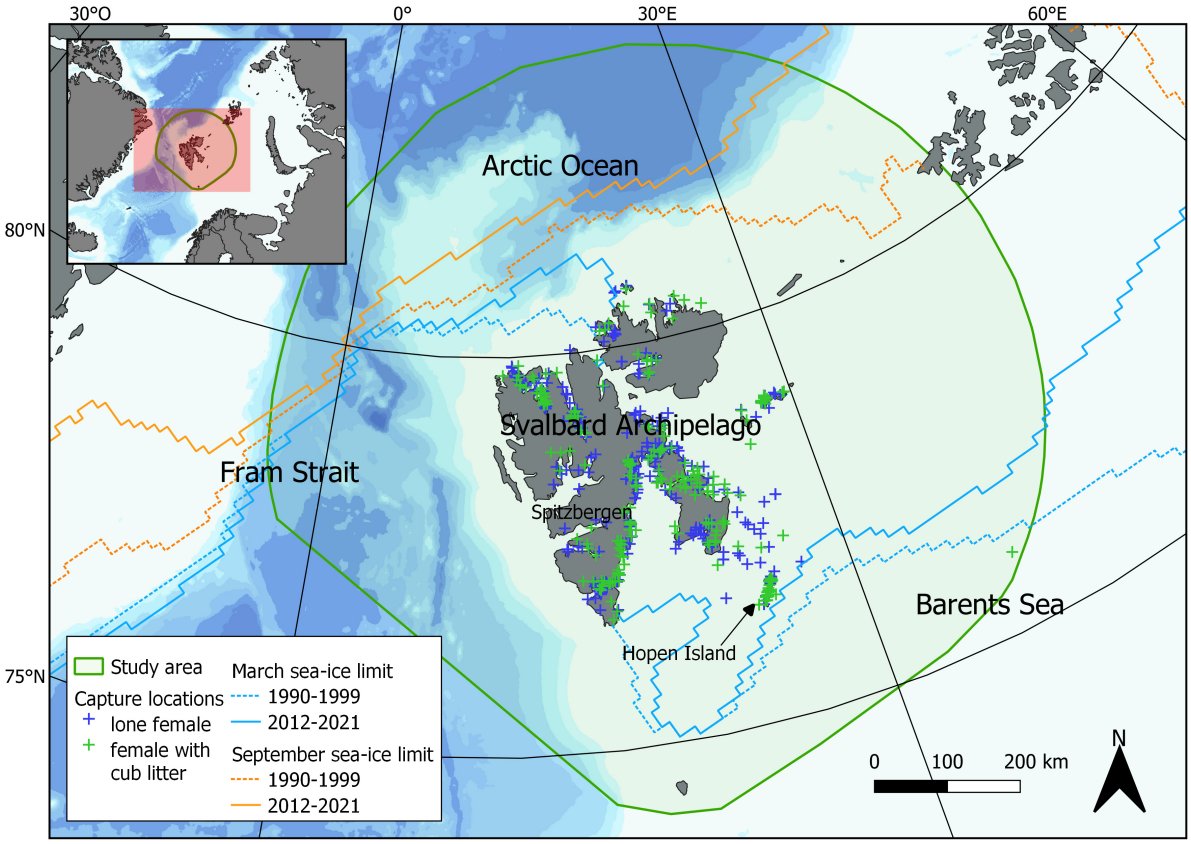


FIGURE 1
Capture locations of adult female polar bears in the Svalbard area from 1992 to 2019 and 2021 to 2022. Captures of lone females from 1992 to 1999 were excluded from our analysis and are not represented. The lines represent the mean maximum (March) and minimum (September) sea-ice extents for the periods 1990–1999 and 2012–2021. The study area corresponds to the 95% minimum convex polygon of telemetry locations of 135 adult females recorded between 1989 and 2021.

and thereby influences sea-ice conditions. When the AO is low, more sea-ice is trapped in the middle of the Arctic by the winds resulting in greater area of multilayer sea-ice (Rigor et al., 2002). When the AO is high, more sea-ice drifts out of the arctic through Fram Strait (Rigor et al., 2002). Nonetheless, the effect of the AO does not appear to be captured by satellite-derived sea-ice metrics, likely because it impacts characteristics of sea-ice other than extent (e.g., thickness, presence of rafter ice, presence of leads) that may affect polar and their prey (Derocher, 2005; Ferguson et al., 2005; Pilfold et al., 2015; Rode et al., 2018b, 2021).

We retrieved a monthly arctic oscillation time series from the NOAA website.[2] We define winter as the period January–March and spring April–June. We calculated the winter and spring AO by averaging the monthly AO index over the corresponding period for each year in the study period.

We expected a high AO index in the winter and the spring of the year preceding capture (*PriorWinterAO*, *PriorSpringAO*), and in the winter of the year of capture (*WinterAO*), to be associated with lower reproductive performance.

## Statistical analyses

We performed all analyses in R version 4.1.2 (R Core Team, 2021) and Rstudio (RStudio Team, 2021). All the figures were made using the ggplot2 (Wickham et al., 2020) and patchwork (Pedersen, 2020) packages.

### Analysis of litter production and litter size

#### Modeling approach

First, we used a binomial Generalized Linear Mixed Model (GLMM) to examine the-effect of individual and environmental covariates on the probability of litter production (i.e., of being with at least one cub at capture) in interaction with age. The covariates were size (*Size*, with a quadratic effect $Size^2$), *DayBreakup*, *DayFreezeUp*, *WinterAO*, *PriorWinterAO*,

2 https://www.climate.gov/news-features/understanding-climate/climate-variability-arctic-oscillation

and *PriorSpringAO*. We also controlled for the date of capture (*DateCapture*). We included data on $n_1$ = 441 captures of females that were alone or with ≥1 cub in 2000–2019 and 2021–2022, corresponding to 289 distinct females. Females with yearlings or 2-year-olds were not included in this analysis. We did not take into account the reproductive status of females in the years prior to capture, meaning we considered that females who were alone in spring t-1 and those who had a litter but lost it have the same probability of producing a litter. Including only females for which past reproductive history was known would have greatly reduced sample size ($n$ = 68 instead of 441). Second, we built another binomial GLMM to investigate the effect of the same individual and environmental covariates on the probability of having a twin or triplet litter, given a litter. In this analysis, we included data on $n_2$ = 251 captures of females with ≥1 cub in 1992–2019 and 2021–2022 corresponding to 204 distinct females. One female was captured once outside of the study area and was excluded from this analysis. We grouped twin and triplet litters because only 3% of females in our dataset were captured with triplets. Hereafter, we refer to both twin and triplet litters as "large litters." We accounted for environmental variation not captured by our covariates using a yearly random effect. We did not include individual identity as a random effect because of the low recapture rate (25% of females captured more than once, mean number of captures per female 1.53 ± 1.16 for the analysis of litter production; 17% and 1.22 ± 0.57 for the analysis of litter size).

We included the effect of the date of capture for three reasons. (i) To account for the departure of 2-year-old from their mother over the fieldwork season that spanned more than a month, which results in an overestimation of reproductive failures if not accounted for in our analysis of litter production (Cubaynes et al., 2021), (ii) to account for den emergence throughout the field season in our analysis of litter production (Wiig, 1998), and (iii) to account for potential offspring loss since cub mortality rate is high (Wiig, 1998; Folio et al., 2019).

We tested for potential dependences between environmental covariates. As our covariates were not normally distributed, we used Kendall rank correlation tests. *DateBreakUp* and *DateFreezeUp* were correlated with one another with $\tau$ = 0.4. Consequently, we did not include them in the same model. All other covariates included were correlated with $\tau < 0.4$.

In both models, we grouped females into age groups because (i) we did not want to impose the shape of the relationship between age and reproductive output and therefore did not consider age as a continuous variable using for example a quadratic function; (ii) we did not want to use non-linear models [e.g., generalized additive model (GAM)] as this would have made exploring the interaction between environmental covariates and age, and interpreting the results in terms of senescence patterns difficult (iii) sample size was insufficient to consider one category per age (as done in Hayward et al., 2013 for instance). In order to determine the appropriate number of age groups and cut-offs between them, we (1) ran GAMs while controlling for the date of capture and (2) since we were particularly interested in senescence, we ran a segmented regression with two segments to estimate the age at onset of senescence (see Appendix 1, **Supplementary Figures 1–4**). Results from these preliminary analyses yielded the following age categories: 5–9, 10–15, 16–20, and ≥21 years for the analysis of litter production and 5–9, 10–15, and ≥16 years for the analysis of litter size. We grouped females aged 16–20 and ≥21 years in the analysis of litter size because of the low number of females ≥21 years ($n$ = 11, **Supplementary Figure 5**). We estimated age-group-specific effects of all covariates.

### Implementation

We fitted all models in a Bayesian framework using the nimble package (de Valpine et al., 2021). Both GLMMs were ran simultaneously as parts of a single nimble model. All continuous covariates were standardized. We used non-informative normal prior distributions for the regression coefficients and a uniform prior distribution for the standard deviation of the random effect. We ran two MCMC in parallel with different initial values, 135,000 iterations and a burn-in of 10,000 iterations. We kept one out of 10 values from each chain. We used the Gelman and Rubin R-hat diagnostic [R-hat < 1.1, (Gelman and Rubin, 1992)] and visual inspection to assess convergence.

### Variable selection procedure

We considered age-specific covariates to be significant if the 89% credible interval (CRI) of their posterior distribution did not overlap with 0. We used 89% CRI rather than 95% CRI following recent recommendations (Kruschke, 2015; McElreath, 2020). Only age-specific covariates that were significant were retained in the final model. Because our dataset was already divided in three or four categories according to female's age with a limited sample size in each category, we did not investigate additional interactions.

### Calculation of additional quantities

Based on the posterior distributions of parameters obtained from the best models for litter production and litter size, we calculated the absolute probability of having a singleton litter and of having a large litter. It was simply obtained as the product of the probability of litter production and the probability of a large litter. This was possible for females aged ≥16 because the two age categories 16–20 and ≥21 years used in the analysis of litter production are nested within the age category ≥16 years used in the analysis of litter size (Appendix 1).

To get insight into the age-related changes in susceptibility of litter production and litter size to environmental conditions, we calculated the age-specific mean effect size of environmental covariates. To do so, we averaged, for each age class, the values from the posterior distributions of all environmental variables, iteration by iteration.

### Analysis of cub and litter survival

#### Capture-recapture model

We estimated individual cub and litter survival in singleton and large litters using CR data collected between 1992 and 2019. We assessed cub and litter survival between the age of 3–4 months and 15–16 months (i.e., between the cubs' first spring and the following one) depending on maternal age. Due to the limited sample size, we could not determine the optimal number of age groups and cut-offs. We therefore used the same age groups as in the analysis of litter size (5–9, 10–15, and ≥16 years) as they require fewer old individuals than those used in the analysis of litter production. To perform this analysis, we adapted a CR model which accounts for the multiple-year dependency between the demographic parameters of individuals belonging to the same family unit as well as the influence of past-reproductive history on female survival and reproductive performance (see Cubaynes et al., 2021).

### Model assumptions

Polar bears captured in Svalbard are a mixture of resident bears, who stay in the Svalbard coastal area, and pelagic bears who follow the marginal-ice-zone as it retreats and advances (Mauritzen et al., 2001). Pelagic bears have a low recapture probability because they mostly remain outside of our study area, as indicated by extensive telemetry records (Norwegian Polar Institute, unpublished data). As recommended by Cubaynes et al. (2021) to avoid underestimating the survival rates of all bears, we only considered resident individuals captured at least twice between 1992 and 2019 in the analysis of cub and litter survival.

Because of identifiability issues due to the relatively low sample size, we could not estimate the probability of litter production for each age class using the CR model. For the same reason, we did not investigate the effect of environmental covariates on cub and litter survival.

### Implementation

We fitted the model in a Bayesian framework using the R2Jags package (Su and Yajima, 2020). We ran two MCMC chains in parallel with different initial values, over 20,000 iterations discarding the first 9,000 iterations. We kept one out of 5 values from each chain. We used the R-hat diagnostic and visual inspection to assess convergence.

## Results

### Litter production and litter size

Our dataset for the analysis of litter production included 441 captures (of 289 females), among which 167 (38%) were females with cubs. Of those 441 captures, 170 (38%) were females aged 5–9 (mean age ± SD: 6.96 ± 1.42 years), 172 (39%) were females aged 10–15 ($\bar{x}$ = 12.3 ± 1.70 years), 63 (14%) were females aged 16–20 ($\bar{x}$ = 17.8 ± 1.44 years), and 36 (8%) were females aged ≥21 ($\bar{x}$ = 23.4 ± 2.38 years). Our dataset for the analysis of litter size consisted in 251 captures (of 201 females), 167 (66%) of which were females with a large litter. Among these 251 captures of mothers, 99 (39%) were females aged 5–9 ($\bar{x}$ = 7.25 ± 1.18 years), 107 (43%) were females aged 10–15 ($\bar{x}$ = 12.4 ± 1.75 years), and 45 (18%) were females aged ≥16 ($\bar{x}$ = 19 ± 2.23 years). None of the 10 females aged 25 or more were accompanied with cubs at capture. The oldest female in our dataset was 29 years old. A more detailed overview of the dataset is provided in Appendix 2 (**Supplementary Table 1**; **Supplementary Figure 5**).

Below we present the results from the models that included the date of sea-ice break-up in the year prior to capture (*DateBreakUp*) as the sea-ice covariate. A full account of the variables included in the final models is provided in **Supplementary Table 2**. The models that included the date of sea-ice freeze-up in the year prior to capture (*DateFreezeUp*) produced similar results that we report in Appendix 3.

### Effect of age

The probability of producing a litter varied with female age (**Figure 2A**). It increased 1.8 folds from age 5–9 to age 15–10. Then it decreased slightly at age 16–20 before declining more sharply after age 20, with females aged ≥21 having less than half as many chances of producing a litter as females aged 10–15 (**Figure 3**). The probability of having a large litter, given a litter, also varied with age, with the same pattern as litter production although the difference between age groups was lower (**Figure 2B**). Females aged 10–15 had 1.2 and 1.4 times as many chances of having a large litter, given a litter, compared to females aged 5–9 and ≥16, respectively (**Supplementary Figure 6**). The absolute probability of having a large litter (i.e., the product of the probability of producing a litter and the conditional probability of having a large litter) peaked at age 10–15 (**Figure 3**). Females aged 16–20 had a slightly lower probability of producing a litter than females aged 10–15, but almost half of their litters were singleton litters.

### Effect of the date of capture

The date of capture influenced both the probability of litter production and the probability of having a large litter (**Figure 2**). The probability of litter production increased over the field season for all females except females aged 10–15 (**Figure 4**). However, for females aged 5–9 (but not for older females), the probability of a large litter decreased over the field season (**Supplementary Figure 7**). Consequently, the absolute probability of having a large litter had a parabolic shape for females aged 5–9, peaking at mid-season (**Figure 4**). By contrast, for females aged 16–20 and ≥21, the absolute

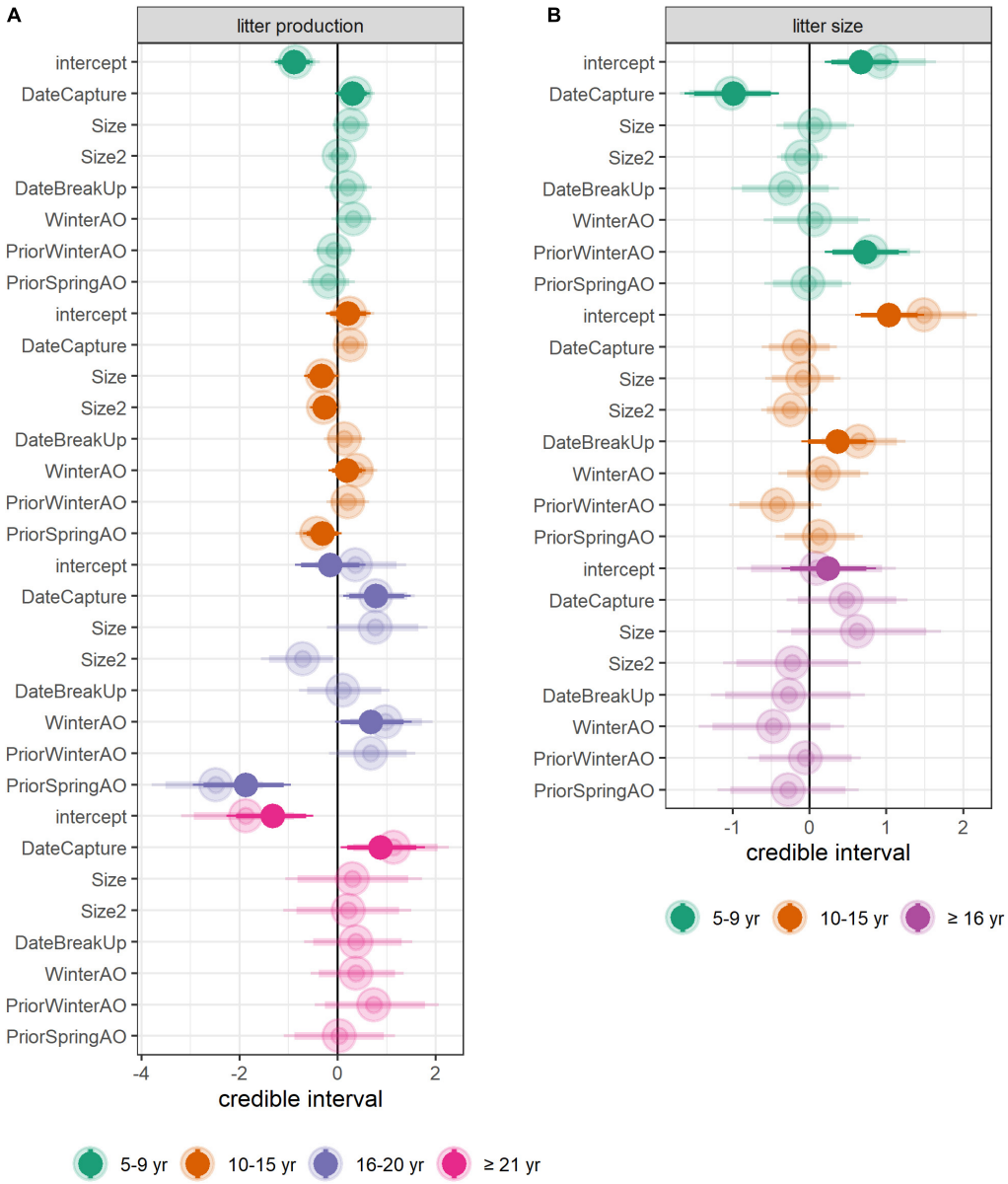


FIGURE 2
Output of the models of litter production and litter size. Posterior distributions of the coefficients in the full model (transparent colors) and in the final model (solid colors) of **(A)** litter production and **(B)** litter size. The mean (dot), 89% CRI (thick line), and 95% CRI (thin line) are provided. Results are on the logit scale. *DateBreakUp* refers to the date of sea-ice break-up in the year prior to capture (see Appendix 3, **Supplementary Figure 8** for the version of this figure obtained when using *DateFreezeUp* as the sea-ice covariate), *WinterAO*, *PriorWinterAO*, and *PriorSpringAO*, respectively refer to the mean Arctic Oscillation index over the winter of the year of capture, over the winter of the year preceding capture and over the spring of the year preceding capture.

probability of having a large litter increased monotonously throughout the season.

## Effect of environmental conditions

### Litter production

A high winter AO index in year t was associated with a higher probability of litter production for females aged 10–15 and 16–20 (**Figure 2A**). A high spring AO index in year t-1 was also associated with a lower probability of litter production for females aged 10–15 and 16–20. The effect was particularly strong for the latter age group (**Figure 2A**). Dates of sea-ice break-up and freeze-up in year t-1 had no significant effect on the probability of litter production although there was a trend for a positive effect of the date of break-up, and a negative effect of the date of freeze-up for all aged age groups (**Figure 2A**; **Supplementary Figure 8A**). Overall, the mean effect size of

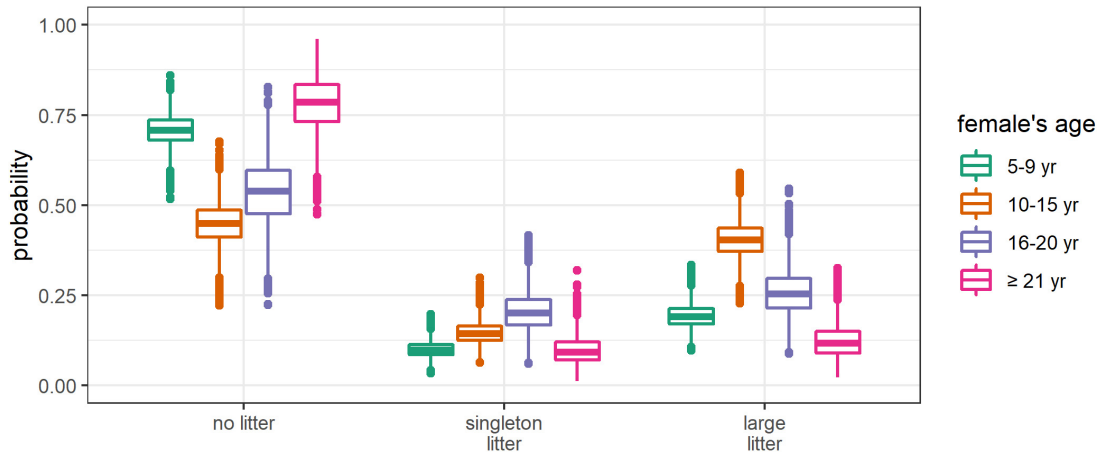


FIGURE 3
Posterior distributions of the absolute probabilities of having no litter, a singleton litter, or a large litter, depending on female's age. All the other covariates were set at their mean value (*DateCapture* = 104, i.e., April 14, *Size* = 195 cm, *DateBreakUp* = 174, i.e., June 23, *WinterAO* = −0.06, *PriorWinterAO* = 0.01, *PriorSpringAO* = 0.12).

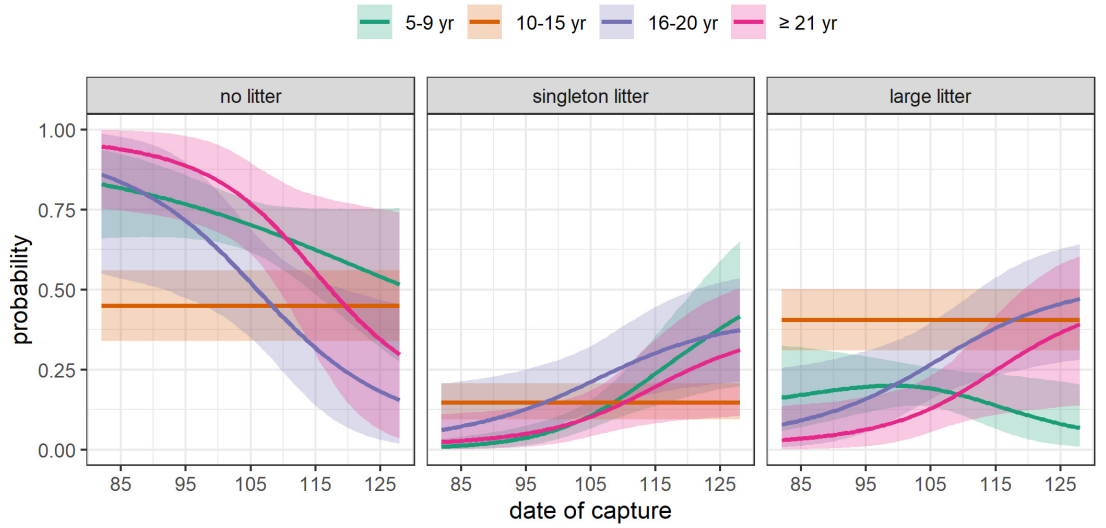


FIGURE 4
Effect of date of capture on the absolute probability of having no litter, a singleton litter, or a large litter, depending on female's age. The mean (solid line) and 95% CRI (shading) are provided. All the covariates other than *DateCapture* were set at their mean value (*Size* = 195 cm, *DateBreakUp* = 174, i.e., June 23, *WinterAO* = −0.06, *PriorWinterAO* = 0.01, *PriorSpringAO* = 0.12).

environmental covariates (including non-significant ones) was comparable for females aged 5–9 and 10–15 but was higher for older females, particularly females aged 16–20 (**Figure 5A**; **Supplementary Figure 9A**).

## Litter size

The date of sea-ice break-up in year t-1 was positively correlated to the probability of a large litter for females aged 10–15 (that is, the later the sea-ice retreated in the year prior to capture, the larger the litter, **Figure 2B**). By contrast, the date of sea-ice break-up in year t-1 had a non-significant negative effect on the probability of a large litter for females aged 5–9 and ≥16 (**Figure 2B**). There was a trend for a negative effect of the date of sea-ice freeze-up in year t-1 (**Supplementary Figure 8B**). The probability of a large litter increased with the winter AO index in year t-1 for females aged 5–9 (**Figure 2B**). Overall, the mean effect size of environmental covariates (including non-significant ones) was comparable across age groups (**Figure 5B**; **Supplementary Figure 9B**).

Since none of the environmental covariates were selected in both binomial regressions for a given age group, we obtained straightforward patterns of absolute probabilities (i.e.,

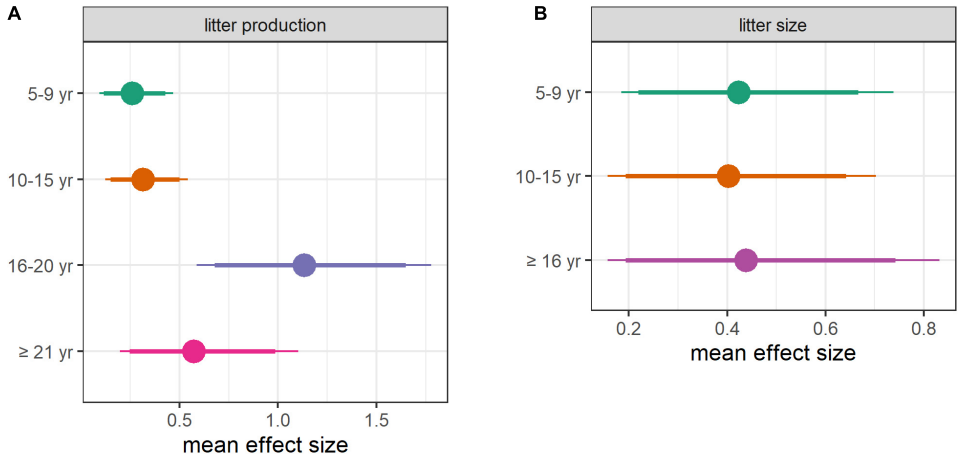


FIGURE 5
Mean effect size of all environmental covariates. Mean effect size of environmental covariates for **(A)** probability of litter production and **(B)** probability of a large litter, depending on female's age. The mean (dot) with 89% CRI (thick line), and 95% CRI (thin line) are provided. Each distribution was obtained by averaging values in the posterior distributions across environmental covariates, iteration by iteration and for each age groups. Posteriors were on the logit scale. The models used for this figure included *DateBreakUp* as the sea-ice covariate (see Appendix 3, **Supplementary Figure 9** for the version obtained when including *DateFreezeUp*).

no environmental covariate increased the probability of litter production while reducing the probability of a large litter, or vice versa) (**Figures 6**, **7**).

## Cub and litter survival

Our dataset consisted in 158 capture histories corresponding to 57 captures of independent juveniles and subadults, 444 captures of adult females, 63 singleton cub litters and 84 large cub litters, 73 yearling litters and 19 2-year-old litters.

Cub survival (from the cubs' first spring to the following one) in singleton litters increased with mother's age with a mean of 0.24, 0.58, and 0.75 for females aged 5–9, 10–15, and ≥16, respectively (**Figure 8**). Cub survival in twin litters also increased with mother age from a mean of 0.43 for females aged 5–9, to 0.54 for females aged 10–15, before reaching a plateau at 0.56 for females aged ≥16. Litter survival followed a similar pattern.

# Discussion

Overall, we found contrasted senescence patterns in the four indicators of reproductive performance of female polar bears. Females aged ≥16 had a lower probability of producing a litter and produced smaller litters, but their offspring first-year survival was higher than that of females aged 10–15. Young females had a lower probability of producing a litter and produced smaller litters with reduced cub survival chances over the field season and over their first year. We also showed that environmental conditions influenced reproductive output of all females, and more so for old females.

## Contrasted patterns of senescence across the four reproductive traits

### Senescence in litter production and litter size

We found a reduction in litter production and litter size for females ≥16 (**Figure 3**), in accordance with previous studies on this subpopulation (Folio et al., 2019) and Hudson Bay bears (Lunn et al., 2016). Senescence in litter size has been reported in other polytocous mammals [in carnivores: e.g., lions (Packer et al., 1998), red fox (Lieury et al., 2017), American minks (Melero et al., 2015), meerkats (Sharp and Clutton-Brock, 2010), in ungulates: e.g., moose (Ericsson et al., 2001), Soay sheep (Hayward et al., 2013)]. Similarly, senescence in litter production has been reported in several mammal species (Packer et al., 1998; Bowen et al., 2006; Sharp and Clutton-Brock, 2010; Melero et al., 2015), including brown bears (Schwartz et al., 2003). Interestingly, females aged 16–20 had only a slightly lower probability of producing a litter compared to females aged 10–15, but this was at the expense of litter size, with a greater proportion of their litters being singletons.

Senescence in litter production and litter size are thought to be a result of lower conception rates, implantation rates and/or higher rates of resorption/miscarriage in old females (Hewison and Gaillard, 2001; Melero et al., 2015; Lieury et al., 2017). In Hudson Bay polar bears, pregnancy rates are highest in young females then decline with age, with the sharpest decline occurring after age 20 (Derocher et al., 1992). Failure to

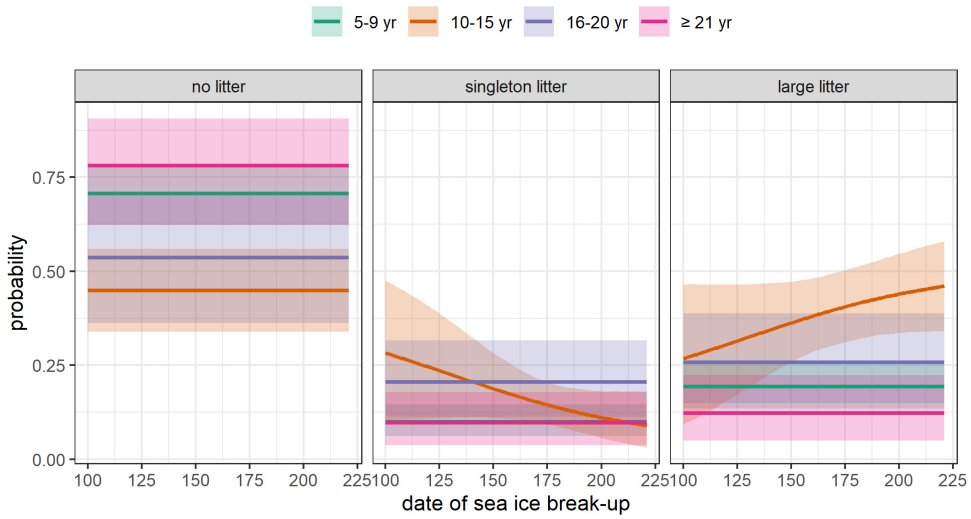


FIGURE 6
Effect of the date of sea-ice break-up in the year prior to capture on the absolute probability of having no litter, a singleton litter, or a large litter, depending on female's age. The mean (solid line) and 95% CRI (shading) are provided. All the covariates other than *DateBreakUp* were set at their mean value (*DateCapture* = 104, i.e., April 14, *Size* = 195 cm, *WinterAO* = −0.06, *PriorWinterAO* = 0.01, *PriorSpringAO* = 0.12).

enter estrous, to produce viable follicle or to implant embryos leading to lower pregnancy rates may account for the lower litter production and size of females in our study, as found in roe deer (Hewison and Gaillard, 2001). Even if old females mate and their eggs implant, they may be more prone to fetal loss (Melero et al., 2015; Lieury et al., 2017). These failures may be driven by the aging of the reproductive physiology, i.e., fertility senescence (Ellis et al., 2018). Alternatively, they may be mediated by senescence in body condition (Nussey et al., 2011). The amount of fat reserves females can accumulate before denning is known to influence reproductive performance, with fatter females being more likely to have cubs in the following spring (Derocher et al., 1992). In southern Hudson bay, old pregnant females are known to be lighter than prime-aged females at den entrance and at den emergence (Derocher et al., 1992; Derocher and Stirling, 1994), suggesting reduced body condition at old age. If such a decline occurs in Svalbard polar bears, it could reduce the ability of old females to conceive or to undergo denning, thereby reducing pregnancy rates and in turn litter production and litter size. In species such as polar bears for which litter size right after parturition is not known, postnatal mortality can lower apparent litter size, and even litter production rate in case of whole litter loss (Derocher et al., 1992). However, we found that in females aged ≥16, litter size did not decline over the field season (**Figure 2B**; **Supplementary Figure 7**). This suggests that old females are capable of providing adequate care to their offspring after den emergence, thereby avoiding litter size reduction and whole litter loss. Alternatively, females aged ≥16 with large litters may emerge from den later, masking litter losses of mothers who emerged early. This would explain the non-significant increase in litter size over the field season we observed (**Figure 2B**). In this case, the effect of the date of capture would reflect the ability of some old females to stay longer in den while keeping both (or all three) of their cubs alive. By contrast, our results suggest that young females struggle to keep all cubs in a large litter alive after den emergence (or to stay in den for longer while keeping their cubs alive), as indicated by the negative relationship between date of capture and litter size (**Supplementary Figure 7**).

Collectively, these findings suggest that lower reproductive output in old females is due to failure in early stages of the reproductive cycle, driven either by fertility or body condition senescence.

There are a few limits to our analysis. First, we did not consider the influence of reproductive status in year t-1 on the probability of producing a litter because including only females for which past reproductive history was known would have greatly reduced sample size. This may have introduced a bias because compared to successful breeders or previously lone females, females who lost a litter in year t-1 are less likely to produce a litter in year t (Cubaynes et al., 2021). While the probability of losing a yearling litter is low, the probability of losing a litter-of-the-year varies between 0.24 (twin litters) and 0.46 (singleton litters) (Cubaynes et al., 2021). Thus the presence of females who lost their litter in year t-1 in our dataset may have led to underestimating the probability of producing a litter. According to our findings, young females were more likely to lose their litter (**Figure 8**), suggesting their probability of producing a litter could have been biased low. However, young females may also be more likely to be nulliparous in year t-1, so it is unclear whether overall, their probability of producing a litter was under- or overestimated. Second, since the reproductive

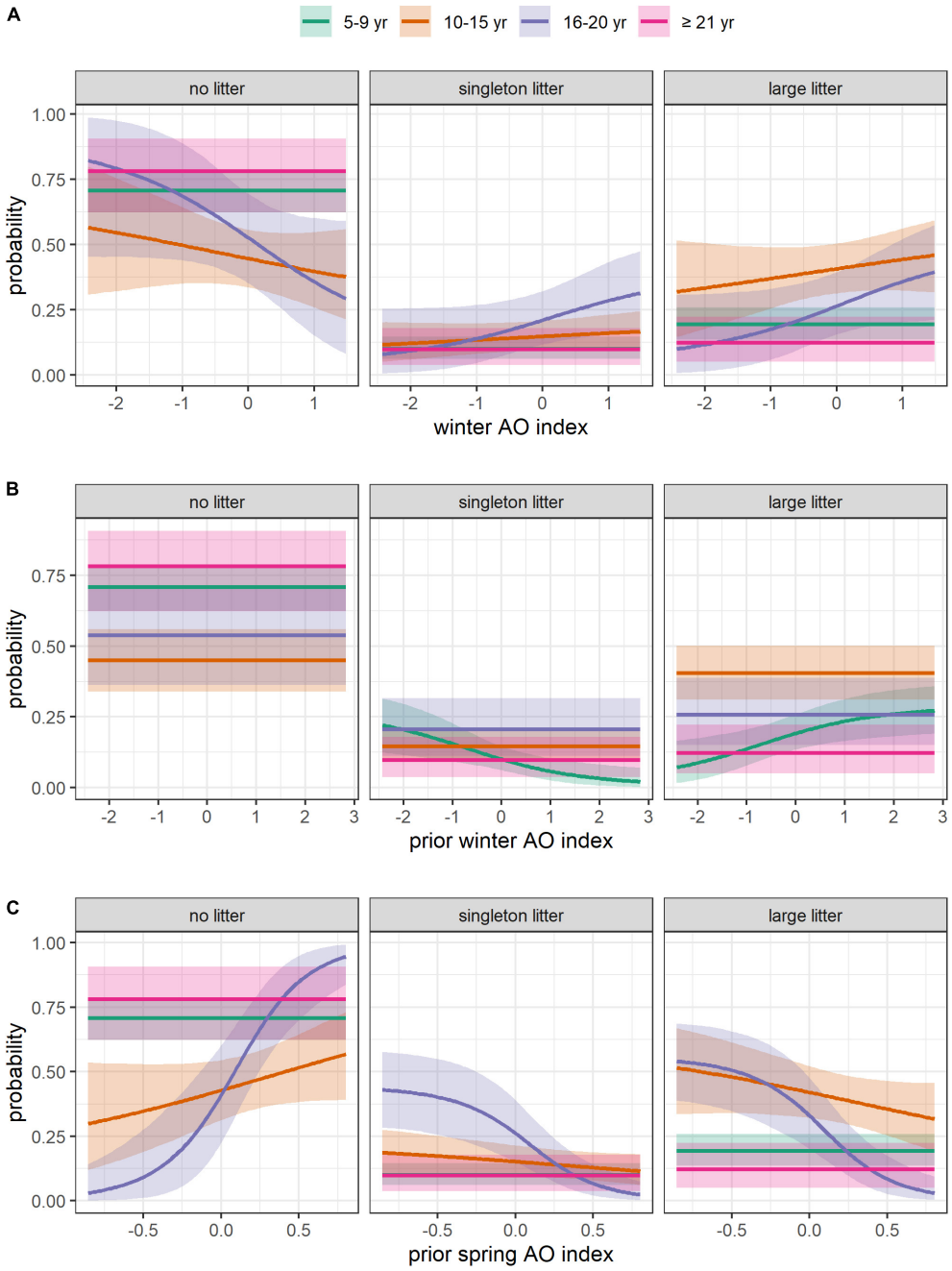


FIGURE 7
Effect of Arctic oscillation on the absolute probability of having no litter, a singleton litter, or a large litter, depending on female's age. **(A)** Effect of the mean AO index over the winter of the year of capture. **(B)** Effect of the mean AO index over the winter of the year preceding capture. **(C)** Effect of the mean AO index over the spring of the year preceding capture. The mean (solid line) and 95% CRI (shading) are provided. All the covariates other than the one represented in each panel were set at their mean value (*DateCapture* = 104, i.e., April 14, *Size* = 195 cm, *DateBreakUp* = 174, i.e., June 23, *WinterAO* = −0.06, *PriorWinterAO* = 0.01, *PriorSpringAO* = 0.12).

cycle of polar bears extends over several years, the number of females who are available to breed (including those who lose their offspring in time to breed) is influenced by environmental conditions experienced over the previous years. It is unclear in what directions these delayed effects of the environment could have biased our results, but they may have obscured the relationships between environmental conditions in year t-1 and the probability of producing a litter. Avoiding these biases would have required using a CR model with memory effects, which is much more complex than our models and requires more data, to

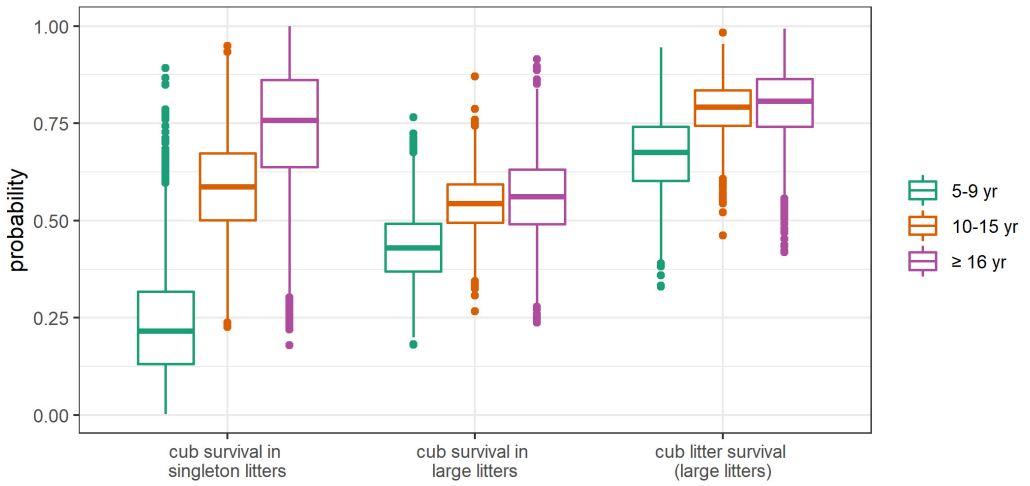


FIGURE 8
Posterior distributions of first-year cub and litter survival probabilities depending on mother's age. First-year survival corresponds to survival between the age of 3–4 months and the age of 15–16 months (i.e., between the cubs' first spring and the following one). First-year litter survival (as opposed to whole litter loss) refers to at least one cub of a large litter surviving.

be able to investigate litter production while accounting for past reproductive history, especially while controlling for the effect of the environment.

## Absence of senescence in cub and litter survival

We did not detect any decline in offspring first-year survival with maternal age (**Figure 8**). This finding is consistent with the absence of a negative effect of the date of capture on litter size in females aged ≥16, further supporting the idea that older females produce fewer offspring or lose them soon after birth but can keep them alive after den emergence. This finding also suggests a weak contribution of maternal-effect senescence in polar bears.

However, the absence of decline in cub survival could result from methodological issues. Firstly, viability selection (or selective disappearance), that is the death of frail, low-quality individuals at an early age leaving only high-quality individuals in old age groups, is known to alter or even mask senescence (Nussey et al., 2008). Due to the relatively low recapture rate in our dataset, we could not include individual identity as a random effect in the GLMMs nor investigate the covariation between survival and parameters related to reproduction in each female in the CR model. However, we found evidence of strong senescence in litter production and litter size, which suggests that viability selection might not be strong enough to mask senescence in our dataset. In addition, we included female structural size in the GLMMs to account for individual quality and longevity as larger individuals usually live longer (Gaillard et al., 2000a) and have higher reproductive success (Derocher and Wiig, 2002; Folio et al., 2019). Importantly, not including size in the GLMMs yielded very similar results (see Appendix 4 for the model without size), further suggesting that the influence of selective disappearance on the observed senescence patterns is limited. Still, we acknowledge the possibility that our approach underestimates the magnitude of the decline in reproductive performance that occurs at old age in polar bears. Secondly, the age of females in the ≥16 age group ranges from 16 to 28, meaning there may be a lot of within-age-group heterogeneity. If only females slightly older than 15 reproduce while older females do not, the high cub survival rate could reflect the performance of the former, instead of the performance of the whole age class. However, very few females live past the age of 20, and those who did continued to produce cubs at least until age 24 (**Supplementary Figure 5**). This indicates that the high cub and litter survival rates do reflect the reproductive performance of the vast majority of females classified as old. There may nonetheless be a decline in cub survival at very old age although detecting it would be challenging given the very few females that reach that age.

Several studies have reported increasing or stable offspring survival or correlates of offspring survival with maternal age [e.g., in mammals (Cameron et al., 2000; Weladji et al., 2006; Hadley et al., 2007; Berger et al., 2015; Oosthuizen et al., 2015), and in birds (Mauck et al., 2012; Ivimey-Cook and Moorad, 2020; Nisbet et al., 2020)]. This pattern can be attributed to three biological processes. (i) terminal investment or allocation, (ii) an increasingly conservative tactic, and (iii) increased experience.

Under the terminal investment hypothesis, reproductive investment (and thereby reproductive cost) should increase with age as an individual's residual reproductive value decreases (Clutton-Brock et al., 1989). For instance, female bottlenose dolphins delay weaning of their last offspring (Karniski et al., 2018), moose give birth to heavier offspring as they age (Ericsson et al., 2001), and old female North American red squirrels

attempt a second reproduction in a given breeding season more often than young females (Descamps et al., 2007). In Svalbard polar bears, direct measures of reproductive allocation are difficult to acquire (e.g., rate of milk and energy transfer to cubs, mother weight loss), and some life-history traits indicative of reproductive allocation in other species show little or no variability (e.g., lactation length, offspring age at weaning, number of breeding attempts per season) (Derocher, 2012). In western Hudson Bay polar bears, Derocher and Stirling (1994) used the ratio of litter mass on mother mass as an indicator of maternal allocation and reported a concave-down relationship with mother's age. This suggests that old female polar bears do not increase allocation-in reproduction to mitigate reproductive senescence.

Another possibility is that old females adopt a conservative reproductive tactic to lower the cost of reproduction by lengthening the interbirth interval and undertaking reproduction only if they have high chances of successfully raising offspring (i.e., if they are in good condition), as do Alpine ibex and southern elephant seals (Rughetti et al., 2015; Desprez et al., 2018). Under this hypothesis, and consistent with our results, litter production and litter size would be lower for old females than for prime-aged females, whereas offspring survival over their first few years would not necessarily be lower.

Finally, old females may benefit from additional experience, simply because they have had more time to accumulate experience (Komdeur, 1996; Mauck et al., 2012) and/or because they have had more breeding attempts (Broussard et al., 2008; Limmer and Becker, 2009; Desprez et al., 2011), which could offset the deleterious effects of maternal-effect senescence on offspring survival (Weladji et al., 2006). A skill that could improve with experience even in late life is habitat selection (Allen et al., 2022). For instance, females with cubs spend less time on active ice and more time on land fast ice compared to lone females (Stirling et al., 1993; Freitas et al., 2012). This is because swimming may be necessary on active ice, putting cubs at risk of hypothermia (Aars and Plumb, 2010), and cub mortality is consequently estimated to be 3.5 times higher on active ice (Reimer et al., 2019). But prey density and vulnerability are thought to be greater on active ice, meaning that females with cubs must weigh the prospect of increased energy intake and transfer to their offspring against the prospect of increased offspring mortality (Reimer et al., 2019). Older, more experienced females may be better able to make the optimal decision.

### Overall reproductive senescence

Regardless of the mechanism underpinning the marked reduction of litter production and litter size in old females and the lack of decline in cub and litter survival, our findings are consistent with Lemaître and Gaillard's prediction whereby senescence of traits involved in late stages of the reproductive cycle should be counter-selected more strongly than senescence in traits involved in early stages (Lemaître and Gaillard, 2017). In polar bears, implantation occurs months after mating, and gestation is thought to have a very small energetic cost as cubs are very small at birth (Derocher, 2012). Losing a cub before birth or after a relatively short period of nursing therefore doesn't represent a major energetic cost. By contrast, losing a cub after a prolonged period of costly lactation likely entails a fitness cost, as it reduces prospects of future reproduction (Cubaynes et al., 2021). This fitness cost could be sizeable even for females aged $\geq$16 because the residual reproductive value of females aged 16 is substantial. Indeed, if a female survived to 24 years of age (our dataset includes 3% of adult females aged $\geq$24), she could wean up to six litters, three of which after age 16.

Nonetheless, according to our findings, the mean probability of a female producing a litter in a given year and of at least one cub reaching its second spring was 0.16 for females aged 5–9, 0.41 for females aged 10–15, 0.37 for females aged 16–20 and 0.18 for females aged $\geq$21. Thus, the increased survival of cubs of females aged $\geq$16 did not offset their reduced litter production and litter size, and reproductive output did decline at old age.

## Effect of environmental conditions

We found that environmental covariates influenced reproductive success in all females (**Figures 2**, **5–7**), in accordance with our expectations (Gaillard et al., 2000b). Indeed, long-lived species are expected to reduce their investment in current reproductive effort or even skip reproduction when environmental conditions are too harsh, in favor of survival and later reproduction (Clutton-Brock et al., 1983; Cubaynes et al., 2011; Lemaître et al., 2015; Desprez et al., 2018).

We found an effect of sea-ice dynamics on the reproductive output of females aged 10–15, with smaller litters following years with early spring break-up (**Figure 6**). Although the effects were not significant, we also found a trend for a positive effect the date of break-up and for a negative effect of the date of freeze-up on litter production (**Figure 2A**). Low sample size associated with wide CRI or the biases mentioned above caused by ignoring potential delayed effects of climate and past reproductive status may have reduced our ability to detect an effect of these covariates. These findings are consistent with previous mechanistic and empirical studies linking reductions in sea-ice availability to declines in reproductive output in other subpopulations (e.g., Rode et al., 2010; Molnár et al., 2011; Lunn et al., 2016; Reimer et al., 2019; Laidre et al., 2020). In the Barents Sea, sea-ice now breaks up in spring 40 days earlier than at the beginning of our study period and freezes up in autumn 55 days later (Stern and Laidre, 2016). Nonetheless, the negative effect of sea-ice loss on population size were not yet perceptible as of 2015 (Aars et al., 2017), perhaps because the population growth rate has not fallen below one yet.

We found that the AO had contrasting effect depending on the season and the time-lag considered (**Figure 7**). Notably, the winter AO in year t and t-1 was positively correlated with litter production and/or size for some age groups. These positive relationships are in contradiction with previous studies conducted in North America showing that a low winter AO index in year t and/or t-1 was associated with higher frequency of predation events by polar bears (Pilfold et al., 2015; Rode et al., 2018b), and higher reproductive output and body condition of females and dependent young (Rode et al., 2021). It's unclear, however, whether the AO has the same effects in Svalbard, although a study conducted in East Greenland found high winter North Atlantic Oscillation (a close relative of the AO) index in year t to be associated with high hair cortisol concentration, an indicator of chronic stress and fasting state (Bechshøft et al., 2013).

We found that spring AO in year t-1 was negatively correlated with litter production of females aged 10–20, in accordance with a previous study conducted in Svalbard (Derocher, 2005). The effect of the AO on polar bears may be mediated by prey availability, as suggested by low ringed seal densities and body condition during or following years with a high NAO index in western Hudson Bay and Svalbard (Ferguson et al., 2005, 2020).

## Interactive effect of age group and environmental conditions

We found that environmental conditions had different effects depending on the age group. A striking example is the effect size of the spring AO index in year t-1, which was six times higher for females aged 16–20 than it was for females aged 10–15. Such results highlight the need to account for potential interaction between age and environmental conditions when investigating age-related variation in demographic traits. While litter size was affected by environmental covariates at a similar level across age groups, litter production of females aged ≥16 was affected more strongly than that of younger females (**Figure 5**; **Supplementary Figure 9**). The reproductive success of old females was thus more sensitive to environmental conditions than that of younger females. From an evolutionary perspective, this finding is in accordance with the canalization theory whereby demographic parameters of individuals contributing less to the population growth rate should exhibit higher variability (Gaillard and Yoccoz, 2003). From an ecological point of view, senescent females are expected to be more vulnerable to environmental harshness (Bunce et al., 2005; King et al., 2005; Pardo et al., 2013). This is supported by the reduced body mass of old females in Western Hudson Bay (Derocher et al., 1992; Derocher and Stirling, 1994). Additional environmental hurdles such as low prey availability may compromise reproduction, lowering the probability of litter production in old females. A conservative tactic whereby old females attempt reproduction only if environmental conditions are favorable may also account for the high susceptibility of litter production to environmental variables. Interestingly, females aged ≥21 appeared to be less susceptible to environmental conditions than females aged 16–20, perhaps because at very old age, reproductive output decline regardless of environmental conditions, potentially as a result of fertility senescence.

It is surprising that we found only a weak impact of environmental conditions on litter production for young females (Gaillard et al., 2000b). Very few females have their first litter at age 5 (i.e., mate at age 4), perhaps because of preferential allocation to growth (Larue et al., 2021), which is still ongoing at that age (Derocher and Wiig, 2002). This allocation tactic may operate independently of environmental conditions, partly accounting for our finding. The reproduction strategy of females aged 6–9 may also be largely independent of environmental conditions, perhaps because of low-quality females delaying reproduction, regardless of environmental conditions (Fay et al., 2016). Another possibility is that the environmental covariates included in our analysis do not adequately represent the environmental conditions that influence litter production for young females. Such factor may operate at spatial and temporal scales that we could not examine in this study [e.g., wind speed over a few days (Pilfold et al., 2015)].

## Perspectives

All females captured in Svalbard since 2011 have been equipped with light and temperature sensors, allowing to detect den entrance and parturition (Friebe et al., 2014; Olson et al., 2017). Further, many adult females have had collars that also provides accurate data on denning (Andersen et al., 2012). Future analyses using these data will allow to differentiate between females who entered den but did not give birth, females who entered den and gave birth but lost their litter, and females who didn't go into denning at all, providing further insight into the drivers of reproductive senescence in female polar bears. These data will also allow to explore potential relationships between denning phenology and litter size and survival (Rode et al., 2018a). Studying age-related changes in female, litter and family mass will also likely improve our understanding of senescence. Finally, data from captive animals may also help assess the timing of senescence in some reproductive traits (e.g., litter size) as well as distinguish between the contribution of body-condition senescence and fertility senescence to reproductive senescence (Henriksen et al., 2005; Roof et al., 2005).

Our study of the aging of several reproductive traits demonstrates differences in the patterns of senescence across traits, emphasizing the need to investigate several indicators of reproductive output to grasp the overall pattern of reproductive

senescence (Massot et al., 2011; Hayward et al., 2013). These differences may arise notably because the strength of natural selection varies depending on when each trait intervenes in the reproductive cycle (Lemaître and Gaillard, 2017), and because of distinct physiological mechanisms driving age-related changes in reproductive performance (i.e., fertility senescence and somatic senescence). Interpreting differences in patterns of senescence across traits in light of evolutionary theory and while considering underlying drivers may foster a clearer understanding of reproductive senescence as a whole. Finally, our study provides insight into how age and environmental conditions interactively determine reproductive output in capital-breeding mammals, highlighting the need to take them into account when investigating reproductive senescence. Comparing senescence patterns in subpopulations differentially affected by climate change may further unravel the interaction between environment and senescence (Kroeger et al., 2018). In turn, a thorough understanding of the age-specific effect of environmental variation will help to accurately forecast demographic responses to climate change.

## Data availability statement

The datasets used in this study are available online at https://doi.org/10.6084/m9.figshare.20749141. R scripts used to generate results and create figures presented in the main text are freely available at https://github.com/MarwanNaciri/Reproductive_senescence_in_polar_bears_in_a_variable_environment.

## Ethics statement

The animal study was reviewed and approved by Norwegian Food Safety Authority and the Governor of Svalbard.

## Author contributions

JA conducted the fieldwork and curated the data. SC and MN designed the study with insight from JA, M-AB, and OG. MN ran the analyses under the supervision of SC and wrote the first draft. All authors contributed to manuscript revisions, read, and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AP declared a past co-authorship with one of the authors JA to the handling editor.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.920481/full#supplementary-material

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EDITED BY
Jean-Francois Lemaitre,
UMR 5558 Biométrie et Biologie Evolutive (LBBE), France

REVIEWED BY
Teri Orr,
New Mexico State University, United States
José E. A. R. Marian,
University of São Paulo, Brazil

*CORRESPONDENCE
Janaina Torres Carreira
jtcarreira@reneco.org

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# Pre-but not post-meiotic senescence affects sperm quality and reproductive success in the North African houbara bustard

Léna Meunier[1,2], Gabriele Sorci[2], Hiba Abi Hussein[1], Yves Hingrat[1], Noé Rehspringer[2], Michel Saint-Jalme[3], Loïc Lesobre[1] and Janaina Torres Carreira[1]*

[1]Reneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates, [2]Biogéosciences, UMR 6282 CNRS, Université de Bourgogne Franche-Comté, Dijon, France, [3]Centre d'Ecologie et des Sciences de la Conservation, UMR 7204 MNHN CNRS-UPMC, Museum National d'Histoire Naturelle, Paris, France

Age-dependent reduction in reproductive success can arise due to multiple factors including a deterioration of reproductive physiology. Senescing males have been shown to produce ejaculates with poor sperm quality, which impinges on male reproductive success. In addition to individual age, gamete age can also affect male reproductive success. Accordingly, variance in male reproductive success can be due to pre-meiotic (referring to individual age) and post-meiotic senescence (sperm age). Here, we tested whether male senescence and sperm cell aging have additive or interactive effects on male reproductive success in a bird with a promiscuous mating system, the North African houbara bustard. To assess the effect of pre-meiotic aging, we compared male reproductive success between two age classes (3-6- and 12–16-year-old). To infer the effect of post-meiotic aging, male ejaculates were collected at three-time intervals following a common initial collection (day 1, 5, and 10). Therefore, day 1 ejaculates are supposed to contain younger sperm than day 5 and 10 ejaculates. Following controlled artificial inseminations, reproductive success was assessed using three fitness-linked traits (hatching success, chick growth rate and survival). In addition to reproductive output, we also assessed whether pre- and post-meiotic aging affected a wide range of sperm and ejaculate traits. In agreement with previous reports, we found that males in the older age class produced less sperm with poorer motility compared to young individuals. However, contrary to the prediction, we found that ejaculates collected at day 5 and 10 tended to have better sperm traits such as motility and velocity. The results on sperm traits were generally mirrored in the effect on reproductive success since young males produced offspring that grew faster and had better survival during the first month of life, and eggs fertilized by sperm collected at day 5 had the highest hatching success. In any of the models, there was evidence for interactive effects of

male and sperm age. Overall, these results confirm the role of pre-meiotic aging on male reproductive success. The lack of evidence for sperm aging could come from the experimental design but might also reflect the pattern of mating frequency in a species with a lek-based mating system.

KEYWORDS

aging, chick survival, reproductive success, sperm age, sperm quality

## Introduction

Senescence is the progressive decline of physiological functions with age, resulting in increased mortality risk (actuarial senescence) and decreased reproductive output (reproductive senescence) (Monaghan et al., 2008). While evidence in supporting senescence to be a widespread phenomenon in nature has been accumulating during the last decade (Nussey et al., 2013), the study of male reproductive senescence has been restricted to a narrower range of species (Lemaître and Gaillard, 2017). Despite this limited taxonomic spectrum, current evidence in humans and captive species of mammals, fish, birds and insects suggests that male age can have profound effects on several traits defining ejaculate and sperm quality (Gasparini et al., 2010; Johnson et al., 2015; Carreira et al., 2017; Herrera-Cruz et al., 2018; Colasante et al., 2019; Fricke and Koppik, 2019; Farooq et al., 2020). For instance, old males have been reported to produce lower ejaculate volume with impaired antioxidant defenses and containing sperm with reduced motility and velocity (Wolf et al., 2000; Sartorius and Nieschlag, 2010; Noguera et al., 2012; Gunes et al., 2016). In addition to these phenotypic changes, old males are also likely to accumulate mutations in the germline due to the continuous cell divisions that are inherent to spermatogenesis (Kong et al., 2012; Thomas et al., 2018). Finally, epigenetic changes of the germline were shown to occur during aging (Immler, 2018; Evans et al., 2019). The combined effect of phenotypic and (epi)genetic changes of sperm due to male age can therefore compromise reproductive success, both by reducing the probability to fertilize the ovum and increasing the risk of embryo/offspring mortality (Hale et al., 2008; Kim et al., 2011; Fay et al., 2016; Lippens et al., 2017; Ruhmann et al., 2018).

Besides these pre-meiotic (organismal) effects of aging, cellular senescence (post-meiotic senescence) can also contribute to determine male reproductive success. Sperm cells are particularly vulnerable to the environmental conditions they are exposed to, and susceptible to oxidative damage (Reinhardt, 2007). Sperm cells are prone to oxidative damage because they (i) produce high amount of reactive oxygen species (ROS) due to their high number of mitochondria and intense metabolic activity, (ii) have limited anti-oxidant defenses due to their reduced cytosol, (iii) do not have known mechanisms of DNA repair, and (iv) have membranes that are rich in unsaturated fatty acids which increase ROS production *via* oxidative carbonyls (Aitken, 2020). After their production during spermatogenesis, spermatozoa are stored in male ducts (epididymis and vas deferens) and after mating they can also be stored in the female reproductive tract in birds (Blesbois and Brillard, 2007). During this period, both in male and female sperm storage, oxidative damage was shown to compromise sperm motility, viability, and DNA integrity in studied species (reviewed by Tarin, 2000); thus, sperm aging can alter the capacity to fertilize the ovum or impair offspring health as shown by White et al. (2008) in kittiwakes (Rissa trydactila). For instance, prolonged sperm storage in male reproductive tract was also associated with decreased velocity and impaired fertilization in the guppy (Poecilia reticulata) (Gasparini et al., 2014, 2017), reduced viability and increased DNA fragmentation in zebrafish (Danio rerio) (Cattelan and Gasparini, 2021) and decreased fertilization, embryo development, hatching and chick condition in kittiwakes (Rissa trydactila) (White et al., 2008). Similarly, sperm aging within the female reproductive tract has been shown to have negative effects on fertility, embryonic development, and hatching rate success in poultry (Nalbandov and Card, 1943; Lodge et al., 1971).

While available evidence convincingly supports the role of both pre- and post-meiotic senescence as important drivers of male reproductive success, a question that has received little attention is whether they have additive or interactive effects on reproductive output (Pizzari et al., 2008). There are several reasons to expect that pre-meiotic senescence might amplify the effect of sperm age. For instance, as the antioxidant defense system decreases with age, sperm produced by old males might be more vulnerable to oxidative damage during the storage period. In agreement with this hypothesis, sperm of old rats are more vulnerable to both *in vivo* and *in vitro* oxidative challenge than sperm from young rats (Zubkova and Robaire, 2006). Such interactive effects of pre- and post-meiotic senescence might have important consequences on male reproductive success, and particularly so in species experiencing strong post-copulatory sexual selection (Birkhead and Pizzari, 2002). Indeed, when females mate with multiple males during the same reproductive bout, sperm compete for ovum fertilization. In this case, when

old individuals compete with young males, they might fail to fertilize the ovum because of a faster rate of sperm senescence. The aim of the present study is to investigate whether pre- and post-meiotic senescence have additive or interactive effects on male reproductive success of the North African houbara bustard (*Chlamydotis undulata undulata*). An interactive effect between pre- and post-meiotic senescence would indicate that any effect of sperm age on sperm quality and/or reproductive success depends on male age. Previous work conducted on this species has shown that male age negatively affects ejaculate quality, hatching success and offspring growth, survival and sperm production (Preston et al., 2011, 2015; Vuarin et al., 2021). Old males also suffer from reduced fertilization success during competitive interactions with young males, even when males of both age classes provide equal number of sperm of similar motility to the insemination (Vuarin et al., 2019a). In addition to this, female houbara bustards can store sperm for weeks, as shown by females being able to lay fertile eggs weeks after being inseminated, and approximately 70% of chicks are sired by the last male used for insemination (L. Lesobre, personal observation). Thus, the houbara bustard appears to be a good candidate to explore the contribution of pre- and post-meiotic senescence on male reproductive success. To this purpose, we artificially inseminated female houbara bustards with sperm collected from old and young males. For each age class, we also collected sperm at different intervals with respect to a common initial sperm collection. A longer interval between the initial collection and the collection used to inseminate the female therefore indicates prolonged sperm storage and therefore older sperm. This experimental design allowed us to assess the effect of male and sperm age on several sperm traits (viability, motility, velocity, morphology, and ROS production) and reproductive success (hatching success, chick growth rate and survival). If pre- and post-meiotic senescence have synergistic effects, we predicted statistically significant interactions between male and sperm age on sperm quality and reproductive output.

# Material and methods

## General procedure and experimental groups

All birds used in this study are part of the Emirates Center for Wildlife Propagation (ECWP), a conservation breeding program located in eastern Morocco, aiming at reinforcing natural populations of the North African houbara bustard (Lacroix, 2003). This program entirely relies on artificial inseminations (Saint Jalme et al., 1994). Males and females on this experiment were born in captivity, are part of ECWP breeders flock and have been used routinely for semen collection and artificial inseminations in the previous seasons. Briefly, semen was routinely collected using a dummy female presented to males. Once the male mounted the dummy and started copulating, a glass dish was used to collect the ejaculate by pressing it gently on the male cloaca during ejaculation. The semen was immediately transferred to a 2 mL cryotube, maintained at room temperature. Ejaculates were directly assessed for motility (using a mass motility index ranging between 0 and 5, see Vuarin et al., 2019a for details), volume and sperm concentration, and diluted (1:1) in Lake 7.1 (Lake and Ravie, 1984).

For the present experiment, a 20 µl aliquot was diluted in 180 µL DPBS-BSA (1.5%) and set aside for flow cytometry analyses (see below), while the remaining was used to inseminate females. We used semen from 307 males belonging to two age classes: 154 young males (mean ± SD = 4.14 ± 1.09, min-max = 3–6 years), and 153 old males (mean ± SD = 13.58 ± 1.61, min-max = 12–16 years). Age categories were chosen based on previous work and senescing patterns (Preston et al., 2011; Vuarin et al., 2019a). These birds were subsequently allocated to three groups (evenly with respect to male age), corresponding to three intervals of sperm collections. Specifically, an initial semen collection was performed (day 0) and then for each group the collections were repeated after 1, 5, or 10 days. All birds experienced the 3 collection intervals, with a rest period of 12 days between cycles (**Supplementary Data** and **Supplementary Figure 1**).

## Semen analysis

Flow cytometry analysis was performed using a Guava easycyte 5HT (IMV, L'Aigle, France) flow cytometer equipped with a 150-mW blue argon laser (488 nm) and 3 color photomultipliers green (525/30 nm), yellow (583/26 nm) and red (695/50 nm). For each sample 10,000 events were counted in the sperm population gate. Data were analyzed using the IMV Easycyte plus software (IMV, L'Aigle, France).

### Viability

Sperm viability (% live out of 10,000 cells) was assessed using Easy Kit viability (IMV, L'Aigle, France), based on propidium iodine (PI) and SYBR-14 dyes. PI enters broken plasma membrane and binds to nucleic acids provoking the emission of 617 nm red fluorescence (Gillan et al., 2005). SYBR-14 dye is membrane permeable and stains only living sperm emitting 520 nm green fluorescence when binding to nucleic acids (Garner et al., 1994). Kit's 96 wells plate were loaded with 180 µL of PBS/1.5% BSA and 20 µL of diluted semen (1:10 in PBS/1.5% BSA). Negative control was performed diluting the sample in distilled water instead of PBS to promote membrane damage. Samples were analyzed after 10 min of incubation in the dark at room temperature.

### Oxidative stress

Intracellular sperm oxidative stress was measured in live cells using a combination of Dihydroethidium (DHE, 2 mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich, St Louis, USA), which emits 606 nm orange fluorescence when reacting with superoxide anions $O_2^-$, a ROS (Zhao et al., 2005), and SYBR-14 (Sigma-Aldrich) to measure percentage of living and oxidized sperm cells (out of 10,000 cells). In a 96 well plate 180 μL of PBS/1.5% BSA, 20 μL of diluted semen (1:10 in PBS/BSA), 1 μL of SYBR-14 (Sigma-Aldrich, St Louis, USA, 1 mM stock solution, 20 μM in DMSO work solution) and 2 μL of DHE (2 mM in DMSO) were added. Negative control was performed incubating sperm with hydrogen peroxide for 15 min at 40°C prior to analysis. Samples were analyzed after 15 min of incubation in the dark, at room temperature.

### Motility

In addition to the initial mass motility index, sperm motility was further evaluated with Sperm Class Analyzer software (SCA, Microptics, Barcelona, Spain). Four microliters of diluted semen (1:10 in PBS/BSA 1.5%) were placed on a Makler chamber under a negative phase contrast microscope (Olympus BX41, 10 × objective) linked to the computer by a camera. A maximum of five fields were analyzed to reach 350 sperm cells, 25 frames on each field were evaluated. Percentage of motile sperm and velocity [curvilinear velocity VCL (μm/s)] were recorded. VCL was selected among other parameters as it refers to the velocity of the sperm real trajectory.

### Morphology

To evaluate sperm morphology, semen was fixed by adding 10 μL of 4% buffered paraformaldehyde to 20μL of semen diluted in PBS/BSA 1.5% (1:10). Then, 6 μl of this solution were placed on a slide with a coverslip (wet mount) and observed under a phase contrast microscope (Olympus BX41, objective ×100, oil immersion). For each sample 100 sperm cells were classified according to 8 categories (Bloom, 1973; Alkan et al., 2002): acrosome loss, abnormal head, swollen head, midpiece defects, tail defects, cytoplasmic droplet, teratogenic sperm (double head or tail) and normal.

## Artificial insemination, egg laying, and chick rearing

A total of 310 captive bred females were inseminated only with one of either 95 young males ($N$ = 177 females, mean female age ± SD = 10 ± 2.4, min-max = 4–13 years) or with 74 old males ($N$ = 133 females, mean female age ± SD = 10.48 ± 2.45, min-max = 3–13 years), from the three semen collection intervals. Females were inseminated with doses containing on average 26.10 million sperms (±16.22), an average inseminated volume of 86.18 μL (± 30.29), and a mass motility index $\geq$ 3.5. The experimental inseminations were performed between the first and the second clutch with no previous insemination during the season, to guarantee the storage tubules were empty and no further insemination were performed before the experiment related eggs were collected.

For each inseminated female, we aimed at collecting the first four eggs laid corresponding approximatively to 2 clutches. The eggs were collected after a single homospermatic insemination. However, not all females laid 4 eggs. Overall, 599 eggs were laid, and more specifically 44 females laid one egg, 246 laid 2 eggs, 17, 3 eggs and 3 laid 4 eggs. Upon laying, eggs were collected and artificially incubated. 54 eggs showed defects and hence were not incubated, resulting in a total of 545 incubated eggs. Eggs were candled at day 8 to check embryo development. When no signs of fertility were detected, they were opened to discriminate early embryonic death from non-fertilized eggs. The percent of successfully hatched eggs (hatching success) was recorded. Body mass of hatchlings was measured daily (to the nearest of 1 g) from day 2 to 60 post-hatching. Any mortality occurring during this period was also recorded. Chicks were reared and manually fed in cages until 35 days and after transferred to outdoor aviaries.

## Statistical analyses

Statistical analyses were performed using R 3.6.0. To investigate the effect of male and sperm age (approximated by the day of ejaculate collection) on sperm quality, we ran linear mixed-effect models [nlme package, (Pinheiro et al., 2021)] or generalized linear mixed-effect models [glmmTMB package, (Brooks et al., 2017)]. These models included male age, the day of collection and their interaction as fixed effects. Moreover, to account for seasonal variation in sperm quality, the date of each ejaculate collection (designated hereafter as "date"), was also included in the models as a polynomial term of degree three since visual inspection showed a non-linear relationship with the response variables. Male identity was included as a random effect to account for dependency between samples collected from the same male at different time of the season.

The effect of male and sperm age on hatching success was assessed using a generalized linear mixed-effect model. For offspring survival, we restricted our analysis to the first 35 days of life since, according to the breeding protocol, chicks are transferred to different breeding facilities and conditions might interfere or mask the genuine effects of father age and sperm age. However, for reference, analyses up to 60 days are reported in **Supplementary Figure 2** and **Supplementary Tables 4, 5**). Chicks' survival was modeled using the mixed effects Cox proportional hazards models (coxme package, Therneau, 2012). Offspring growth rate up to day 60 was modeled using a linear mixed-effect model. Preston et al. (2015) showed an effect

of mother age on hatching success, therefore we decided to include it in these models as a quadratic fixed effect term in addition to male and sperm age, and hatching date (to correct for possible variation across the season). For hatching success and survival models, female and male identities were used as crossed intercept random effects to account for dependency between siblings, while the female and individual identities were used as nested intercept random effects in the offspring growth rate model to account for dependencies between siblings and repeated measures of the same chick. Finally, the model on offspring growth rate also included offspring age (and squared age), offspring sex, and the interactions between male and offspring age as additional fixed effects, to account for any potential difference in growth rate between sexes and father age groups.

Model families were chosen based on the type of response variable and its distribution. Stepwise backward elimination approach based on the corrected Akaike Information Criterion (Mazerolle, 2006) was used to select the best parsimonious model (the model with the lowest AICc) (Arnold, 2010; Aho et al., 2017). The model assumptions regarding the normality of residuals, homoscedasticity, and independency were checked. In case of assumption violation, data transformation, within-group variability and/or autocorrelation modeling were performed. The marginal effects plots based on the models were generated using the ggeffects R package (Lüdecke, 2018). Differences in sample size across models are due to missing values. More details about the model building and selection are given in **Supplementary material**.

## Ethical statement

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534–98), providing control of the wellbeing of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled.

# Results

## Ejaculate attributes and sperm traits

Both male and sperm age affected volume and sperm number in the ejaculate. Young males produced ejaculates with larger volume and higher number of sperm (**Table 1** and **Figures 1A,B**). Ejaculates collected after a longer interval (day 5 and 10) from the common collection also had larger volume and more sperm (**Table 1** and **Figures 1A,B**), suggesting that the sperm population kept accumulating with time. The interaction between male and sperm age (day of collection) was not significant and was not retained in the selected most parsimonious model, neither for ejaculate volume nor for number of sperm in the ejaculate.

Young males also had a higher proportion of motile sperm in the ejaculate compared to old males (**Table 1** and **Figure 1C**), and higher sperm curvilinear velocity (VCL) (**Table 1** and **Figure 1D**). As for ejaculate volume and number of sperm, both proportion of motile sperm and sperm velocity were higher in ejaculates collected at day 5 and 10 (**Table 1** and **Figures 1C,D**).

Male age was not retained in the most parsimonious selected models for the percentage of sperm with normal morphology, the percentage of dead sperm, and the percentage of DHE positive sperm per ejaculate (**Table 2** and **Figure 2**). However, the percentage of sperm with normal morphology increased as the collection interval increased (**Table 2**). Similarly, the percentage of dead sperm per ejaculate was lower when ejaculates were collected after the longest interval from the initial collection (**Table 2** and **Figure 2A**). However, the percentage of DHE positive sperm (a measure of oxidative stress) increased with day of collection, suggesting higher oxidative stress in sperm with prolonged storage time (**Table 2** and **Figure 2B**).

## Reproductive success

Over the 545 eggs that were incubated, 393 hatched (72.11%). Hatching success did not differ according to male age (however, older females laid eggs with lower hatching success) (**Table 3** and **Figure 3A**). Females inseminated with samples collected at day 5 had the highest hatching success (**Table 3** and **Figure 3A**). The interaction between male and sperm age was not significant and not retained in the most parsimonious model. The delay between insemination and egg laying was similar for females inseminated with ejaculates collected at the 3 different intervals (**Supplementary Data** and **Supplementary Table 2**; Day 1 mean ± SD = 6.97 ± 2.23 days, Day 5 mean ± SD = 7.88 ± 2.17 days, Day 10 mean ± SD = 7.62 ± 2.01 days).

Offspring sired by old males had a slower growth rate during the first 60 days compared to offspring sired by young males, as shown by a significant interaction between male and offspring age (**Table 3**). Offspring sired by young males gained on average 0.5 g per day more than those sired by old males, which resulted in a weight difference of 27 g at 60 days [mean weight of chicks sired by old males = 736.37 g (SE = 6.60, CI:723.44, 749.30) for females and 915.68 g (SE = 5.75, CI: 900.43, 930.23) for males; mean weight of chicks sired by young males = 763.62 g (SE = 7.61, CI:752.05, 774.59) and 942.60 g (SE = 6.84, CI:928.89, 955.67) for females and males, respectively]. Neither sperm age alone, nor the interaction between father and sperm age were significant predictors of offspring growth rate.

TABLE 1 Selected parsimonious linear mixed models exploring the effect of male and sperm age on the ejaculate volume, the number of sperm in the ejaculate, the percentage of motile sperm (on the logit scale), and the curvilinear velocity (VCL).

| Response variable | Fixed effect | Estimate | 95% CI | *t*-value | *P*-value |
|---|---|---|---|---|---|
| Ejaculate volume $N = 665$ | Intercept | 8.33 | 7.93, 8.73 | 40.69 | < 0.001 |
| | Male age (young) | 0.63 | 0.14, 1.12 | 2.53 | 0.011 |
| | Collection day (5) | 0.48 | 0.12, 0.84 | 2.63 | 0.009 |
| | Collection day (10) | 0.36 | 0.02, 0.69 | 2.08 | 0.038 |
| | Date | −5.22 | −9.13, −1.31 | −2.61 | 0.009 |
| | Date$^2$ | −5.56 | −9.33, −1.79 | −2.88 | 0.004 |
| Number of sperm in the ejaculate $N = 593$ | Intercept | 3.22 | 3.08, 3.37 | 43.50 | < 0.001 |
| | Male age (young) | 0.42 | 0.27, 0.58 | 5.32 | < 0.001 |
| | Collection day (5) | 0.48 | 0.36, 0.60 | 7.78 | < 0.001 |
| | Collection day (10) | 0.74 | 0.63, 0.85 | 13.05 | < 0.001 |
| | Date | 1.28 | 0.06, 2.49 | 2.05 | 0.040 |
| | Date$^2$ | −2.66 | −3.80, −1.53 | −4.59 | < 0.001 |
| | Date$^3$ | −1.04 | −2.21, 0.11 | −1.78 | 0.0757 |
| Motile sperm (%) $N = 658$ | Intercept | 1.15 | 0.95, 1.35 | 11.15 | < 0.001 |
| | Male age (young) | 0.24 | 0.01, 0.46 | 2.03 | 0.0434 |
| | Collection day (5) | 0.61 | 0.39, 0.82 | 5.50 | < 0.001 |
| | Collection day (10) | 0.56 | 0.36, 0.76 | 5.41 | < 0.001 |
| | Date | −5.45 | −7.73, −3.16 | −4.66 | < 0.001 |
| | Date$^2$ | 0.59 | −1.63, 2.81 | 0.52 | 0.604 |
| | Date$^3$ | −3.44 | −5.74, −1.15 | −2.93 | 0.003 |
| VCL $N = 658$ | Intercept | 31.76 | 30.46, 33.07 | 47.52 | < 0.001 |
| | Male age (young) | 1.80 | 0.39, 3.20 | 2.51 | 0.012 |
| | Collection day (5) | 2.80 | 1.27, 4.30 | 3.61 | < 0.001 |
| | Collection day (10) | 2.56 | 1.13, 3.98 | 3.51 | < 0.001 |
| | Date | −36.38 | −52.20, −20.56 | −4.50 | < 0.001 |
| | Date$^2$ | 49.07 | 33.62, 64.53 | 6.21 | < 0.001 |
| | Date$^3$ | 60.73 | −76.56, −44.89 | −7.50 | < 0.001 |

In total, 667 sperm samples were analyzed. We report the parameter estimate with the 95% confidence intervals, *t*- and *p*-values. See text and online **Supplementary material** for details on the initial model structure and model selection. N, Number of observations; CI, Confidence Intervals. Significance level: $p$-value $\leq 0.05$. Numbers in superscript indicate the polynomial degree of the term.

Twenty-one chicks out of 393 hatchlings died during the first 35 days of life. Offspring sired by young males had a better survival rate compared to offspring sired by old males (**Table 3** and **Figure 3B**). A higher mortality rate was mainly seen in the first 3 days of chicks' life. Neither sperm age, nor the interaction between male and sperm age affected offspring survival.

## Discussion

The aim of the present study was to investigate pre- and post-meiotic senescence and their potential interaction in males of a bird species with a promiscuous mating system, the houbara bustard. In agreement with previous work conducted on the species, we found that pre-meiotic effects are important determinants of ejaculate and sperm quality, and male reproductive success. However, contrary to our prediction, we found that prolonged sperm storage did not impair sperm quality and did not reduce reproductive success, in both young and old males. Thus, we did not find any evidence suggesting that male age might accelerate post-meiotic senescence.

The deleterious effect of male age on sperm quality and reproductive success was expected as it has already been reported in the houbara bustard and other species. Ejaculate attributes such as ejaculate volume, sperm number and mass motility index have been previously shown to increase during the first years of life up to the age of 5–6 years and then gradually decline (Preston et al., 2011). Here, we confirmed these previous findings since old males (mean age of 13.58 years old) produced ejaculates with lower volume and with less sperm. We extended previous findings by using flow cytometry and computer-assisted techniques to assess several sperm traits that collectively contribute to define sperm quality. We found that young males had a higher percentage of motile sperm and sperm with higher velocity (VCL) compared to old individuals. However, we did not find evidence suggesting that male age affected

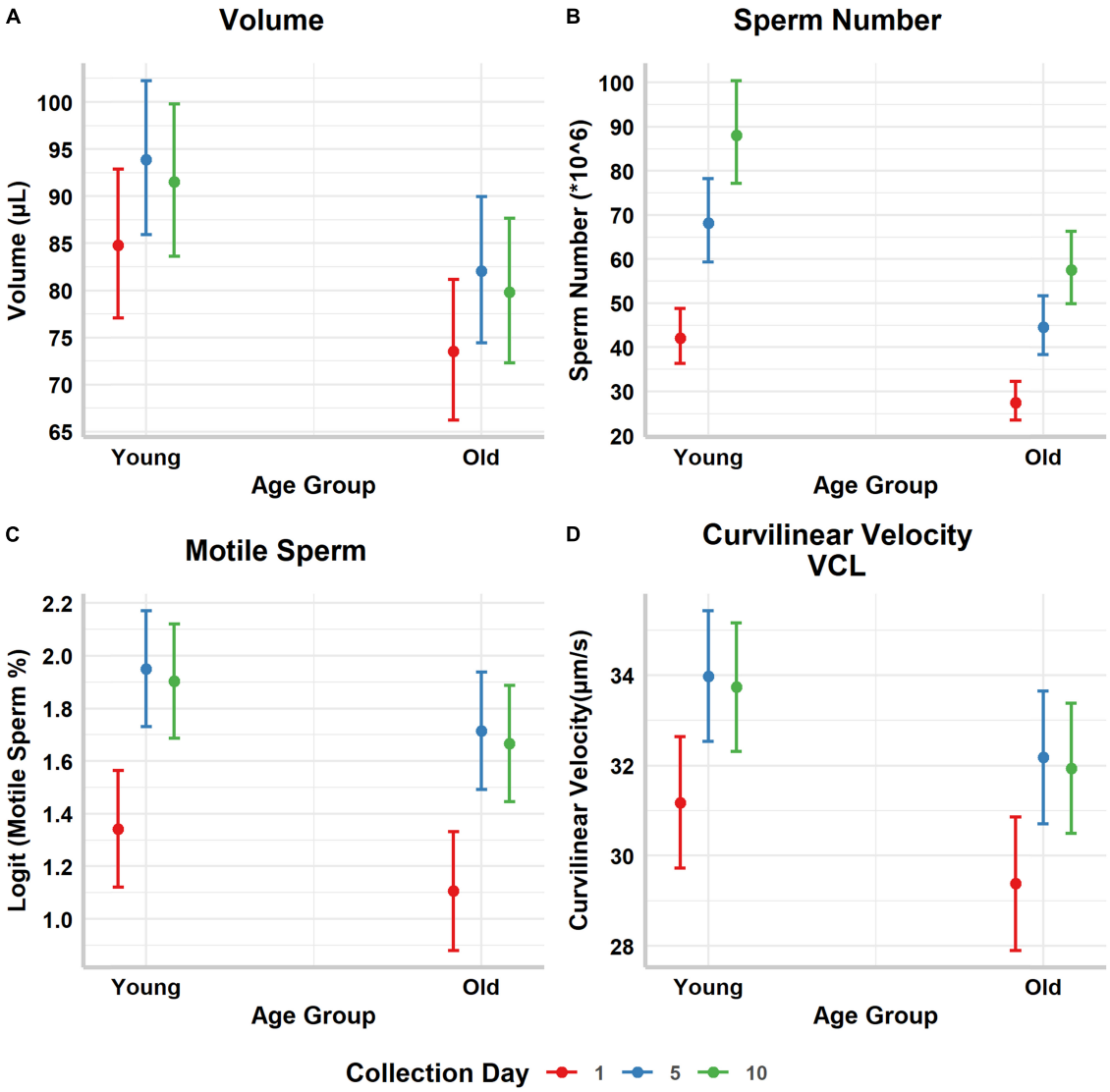


FIGURE 1
Predicted marginal means and their corresponding 95% confidence intervals of the **(A)** ejaculate volume (µL), **(B)** number of sperm in the ejaculate ($\times 10^6$), **(C)** percentage of motile sperm (on the logit scale), and **(D)** curvilinear velocity, per male (old vs. young) and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

other traits related to sperm quality such as the percentage of dead sperm or the percentage of DHE positive sperm in the ejaculate corresponding to the intercellular ROS production, a marker for oxidative stress. These results suggest age-specific effects on different components of sperm and ejaculate quality, similar to the observation that different phenotypic traits have different rates of age-dependent decline (e.g., Lecomte et al., 2010; Hayward et al., 2015).

In addition, to reducing ejaculate and sperm quality, father age has also been shown to affect negatively both offspring growth (Preston et al., 2015) and post-release offspring survival (Vuarin et al., 2019a) in the houbara bustard. Here, we also found that offspring sired by old males had a slightly slower growth rate during the first 60 days of life and a higher mortality rate during the first 35 days of life in captivity, indicating high fitness cost due to reproductive senescence in this species. Despite the statistical significance of the difference in growth rate between paternal age groups, its biological relevance and impact on fitness should be further investigated, especially in the wild.

Contrary to our prediction, post-meiotic aging did not impair sperm quality and reproductive success. We used a proxy of post-meiotic aging, since we compared ejaculates that were collected at different intervals with respect to a common initial collection: the longer the interval, the longer the storage period, and we assumed that a longer storage would increase

TABLE 2 Selected parsimonious linear mixed models exploring the effect of male and sperm age on the percentage of normal, dead, and DHE positive sperm.

| Response variable | Fixed effect | Estimate | 95% CI | *t*-value | *P*-value |
|---|---|---|---|---|---|
| Normal sperm (%) $N = 650$ | Intercept | 0.52 | 0.41, 0.63 | 9.13 | < 0.001 |
| | Collection day (5) | 0.33 | 0.21, 0.45 | 5.22 | < 0.001 |
| | Collection day (10) | 0.43 | 0.31, 0.55 | 7.20 | < 0.001 |
| | Date | −2.11 | −3.44, −0.77 | −3.10 | 0.002 |
| | Date$^2$ | −3.05 | −4.34, −1.76 | −4.65 | < 0.001 |
| Dead sperm (%) $N = 666$ | Intercept | 2.07 | 1.98, 2.17 | 42.37 | < 0.001 |
| | Collection day (5) | −0.05 | −0.17, 0.07 | −0.74 | 0.460 |
| | Collection day (10) | −0.25 | −0.36, −0.13 | −4.28 | < 0.001 |
| | Date | 1.43 | 0.12, 2.73 | 2.14 | 0.033 |
| | Date$^2$ | 2.81 | 1.55, 4.07 | 4.37 | < 0.001 |
| | Date$^3$ | −1.85 | −3.16, −0.54 | −2.78 | 0.006 |
| DHE (%) $N = 667$ | Intercept | 1.05 | 0.88, 1.22 | 12.04 | < 0.001 |
| | Collection day (5) | 0.20 | −0.01, 0.41 | 1.83 | 0.068 |
| | Collection day (10) | 0.45 | 0.25, 0.65 | 4.43 | < 0.001 |
| | Date | −7.71 | −10.00, −5.42 | −6.60 | < 0.001 |
| | Date$^2$ | 0.90 | −1.30, 3.11 | 0.80 | 0.422 |
| | Date$^3$ | −2.25 | −4.55, 0.05 | −1.92 | 0.056 |

We report the parameter estimate with the 95% confidence intervals, *t*- and *p*-values. See text and online Supplementary material for details on the initial model structure and model selection. N, Number of observations; CI, Confidence Intervals. Significance level: $p$-value $\leq 0.05$. Numbers in superscript indicate the polynomial degree of the term.

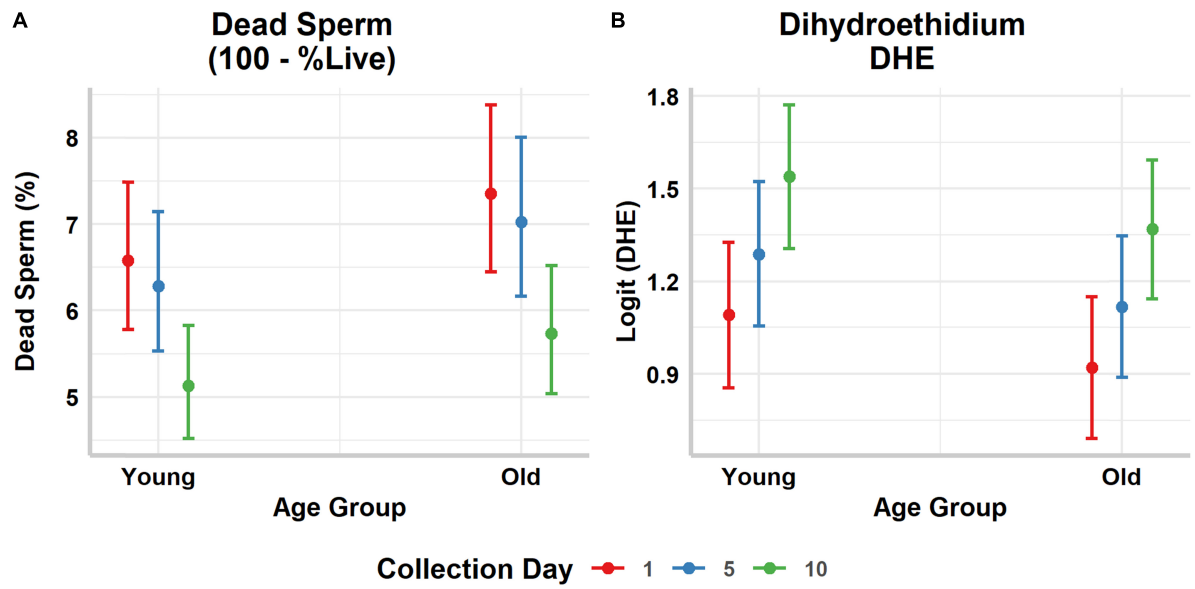


FIGURE 2
Predicted marginal means and their corresponding 95% confidence intervals of the **(A)** percentage of dead sperm, **(B)** and the percentage of DHE positive sperm (on the logit scale), per male (old vs. young) and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

the proportion of old sperm in the ejaculate. Similar reasoning was used in other studies to assess the effect of sperm age on reproductive success. For instance, White et al. (2008) equipped male kittiwakes with rings on the cloaca preventing sperm transfer during mating. Some birds wore the rings for few days, others up to 2 weeks. Once the rings were removed, birds were able to copulate again, and the longer the period of ring wearing the older the sperm transferred. Using this elegant experimental design, they found that sperm aging indeed impaired male reproductive success, in terms of hatching success and hatchling

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TABLE 3 Selected parsimonious generalized mixed linear models exploring the effect of male and sperm age on hatching success, offspring survival and growth rate.

| Response variable | Fixed effect | Estimate | 95% CI | *t*-value | *P*-value |
|---|---|---|---|---|---|
| Hatching success $N = 545$ | Intercept | −0.97 | -2.71, 0.77 | −1.09 | 0.276 |
| | Collection day (5) | 0.88 | 0.95, 1.46 | 2.95 | 0.003 |
| | Collection day (10) | 0.45 | −0.05, 0.96 | 1.76 | 0.078 |
| | Date | 0.03 | 0.01, 0.04 | 3.17 | 0.001 |
| | Mother age | −0.08 | −0.17, 0.01 | −1.73 | 0.084 |
| Offspring survival (HR) $N = 393$ | Father age (young) | 0.27 | 0.10, 0.72 | −2.60 | 0.009 |
| | Date | 568.26 | 0.37, 8.66*$e^5$ | 1.70 | 0.090 |
| | Date$^2$ | 13041.13 | 19.29, 8.82*$e^6$ | 2.85 | 0.004 |
| Offspring growth rate $N = 379$ | Intercept | 199.54 | 201.76, 197.92 | 175.95 | < 0.001 |
| | Father age (young) | 3.35 | 0.98, 5.72 | 2.79 | 0.0058 |
| | Offspring sex (female) | −19.78 | −22.07, −17.49 | −17.09 | < 0.001 |
| | Offspring age | 16523.03 | 16289.47, 16756.59 | 138.61 | < 0.001 |
| | Offspring age$^2$ | 2621.11 | 2507.88, 2734.32 | 45.36 | < 0.001 |
| | Offspring age: Father age (young) | 466.67 | 230.56, 702.77 | 3.87 | 0.0001 |
| | Offspring age$^2$: Father age (young) | 139.71 | 24.55, 254.88 | 2.38 | 0.0175 |
| | Offspring age: Offspring sex (female) | −2907.43 | −3146.17, −2668.70 | −23.86 | < 0.001 |
| | Offspring age$^2$: Offspring sex (female) | −1107.05 | −1222.64, −991.47 | −18.77 | < 0.001 |

We report the parameter estimate with the 95% confidence intervals, *t*- and *p*-values. See text and online **Supplementary material** for details on the initial model structure and model selection. The chicks for which the sex remained unknown were excluded from the data set of the offspring growth rate model. Numbers in superscript indicate the polynomial degree of the term.

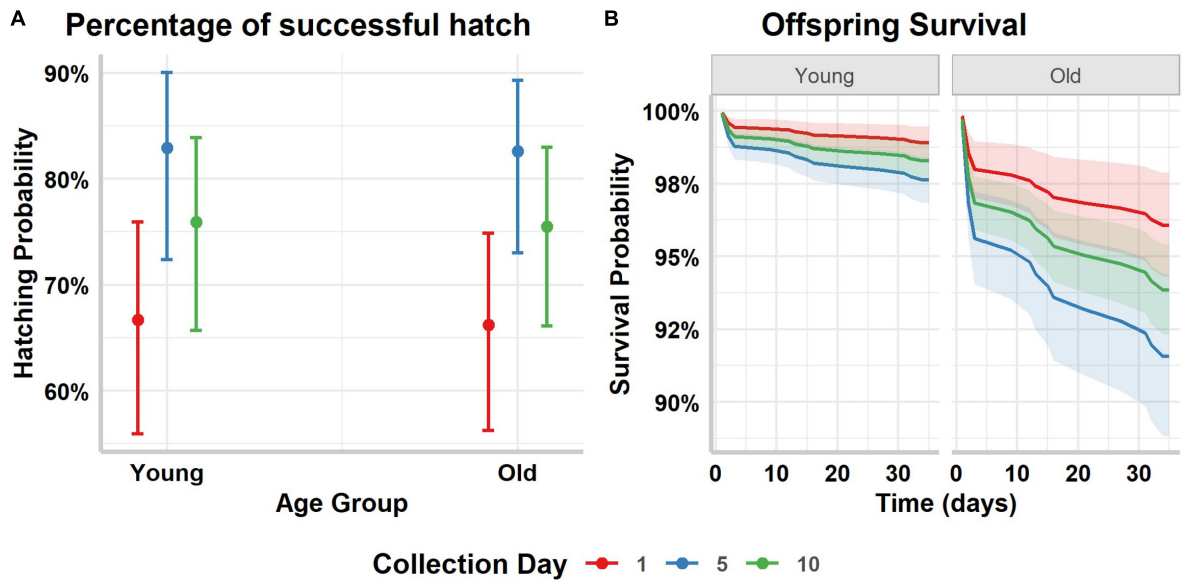


FIGURE 3
Predicted marginal means of the **(A)** hatching success and the corresponding 95% confidence intervals, **(B)** and chick survival during the first month of life and the corresponding standard errors, per male and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

condition (White et al., 2008). In a more recent study, sperm of male zebrafish were initially collected, then males were isolated during 4, 7, or 12 days (sexual rest), after which their sperm were collected again, imposing sexual rest and consequently sperm storage in males, resulting in reduced sperm velocity (Cattelan and Gasparini, 2021). However, it should be fully acknowledged that other studies have failed to report the predicted effect of sperm age on sperm quality. For instance,

Firman et al. (2015) did not find any evidence of decreased sperm quality in house mice (*Mus domesticus*) experiencing a 2-month sexual rest compared to sexually active males. Even within species, the effect of sperm age might be inconsistent since, contrary to Cattelan and Gasparini (2021), a previous work conducted also on zebrafish found that old (stored) sperm had higher velocity compared to freshly produced ones (Vega-Trejo et al., 2019).

Despite an experimental design similar to those adopted in previous studies, we did not find evidence for deleterious effects of sperm aging. Ejaculates collected at day 5 and 10 had higher volume and contained more sperm compared to ejaculates collected at day 1, showing that sperm accumulated in the epididymis and vas deferens over time, in the absence of mating opportunities. However, unexpectedly, the increase in sperm age resulted in a higher proportion of motile sperm in the ejaculate, higher velocity, higher proportion of sperm with normal morphology, and less dead sperm in the ejaculate. The only phenotypic trait potentially suggesting a possible decline in sperm quality with storage was the higher proportion of DHE positive sperm. However, this did not produce any deleterious effect in terms of male reproductive success since eggs fertilized with sperm after a prolonged storage had a higher hatching success and sperm age did not have any effect on chick growth rate and survival.

There are several possible explanations for these results. It is possible that our experimental design failed to produce a substantial change in the age distribution of sperm. The effect of sexual abstinence has been extensively studied in humans (Ayad et al., 2018), and recently, Okada et al. (2020) found an increase on DHE positive cells in male ejaculates with a 4-day abstinence in comparison to 1 day. They suggested that this increase would appear when sperm surpass the physiological necessary epididymal transit. The distribution of sperm age at a given time accumulated in male tract will depend on a differentiation/death process, where new sperm are produced, age and die. In the absence of sperm release (during sexual rest), the variance in sperm age will increase, with the occurrence of old and new sperm in the subsequent ejaculate. This could explain an increase in volume and sperm number along with some level of substitution of dead by viable sperm cells with time, partially explaining why the percentage of dead sperm and defective cells is improved with storage in our study. Physiological loss of sperm cells in the male tract to the cloaca and by duct cells absorption in birds is expected at some rate, however, in domestic fowls it needed more than 4 weeks of storage to observe dead sperm cells being processed by the duct structure (Tingari and Lake, 1972). Nothing is known about such process in bustards, if it happens and at which rate. With this respect, it should also be noted that the longest rest duration in our experimental design corresponds to the average duration of spermatogenesis in birds [ca 10 days in Japanese quails (*Coturnix japonica*), 12 days in barbary drakes, 13–15 days in domestic fowls (Jones and Lin, 1993)]. How sperm are stored in the male reproductive tract can have profound consequences on the reproductive success of males (Reinhardt, 2007). Moreover, if the number of sperm released in the ejaculate is large enough, the reproductive success might still be ensured by the young sperm present in the ejaculate. This also highlights the possible role of post-copulatory selection (including cryptic female choice) as a filter promoting younger sperm within the ejaculate to fertilize the ovum. Finally, it could be that sperm aging occurring post-mating in the female reproductive tract might obscure any effect of prolonged storage in the male reproductive tract. Particularly, this can occur if the rate of sperm aging follows a non-linear (accelerating) trend. To explore this issue, we compared the delay between insemination and egg laying (a proxy of the time sperm spent in the female reproductive tract) and found no difference between the experimental groups (the delay between insemination and egg laying being around 7 days in the three sperm age groups, see **Supplementary material**). This suggests that any additional effect of sperm aging in the female reproductive tract was constant across experimental groups. According to the literature sperm stays quiescent inside the females STT, losing motility and only being released for fertilization of the ovulated oocyte (Sasanami et al., 2013). Molecular exchanges between sperm membrane and STT/female tract fluids do occur, and lactic acid concentrations for example play an important role on sperm release and retention (Matsuzaki et al., 2015). Moreover, molecules of the *utero* vaginal junction involved in sperm storage have been identified and have shown to improve sperm viability *in vitro* when added to the diluent (Matsuzaki et al., 2020) but no evidence of sperm remodeling or cell maturation *per se* were described so far. However, once more, little is known about avian species and especially wild models.

Alternative explanations for our findings refer to the process of post-testicular sperm maturation. In mammals, sperm released in the lumen of the seminiferous tubules at the end of spermatogenesis are not able to fertilize the ovum, yet. During their transit through the epididymis, sperm undergo several morphological and biochemical changes such as nuclear compaction, modification of the membrane composition, loss of cytoplasmic droplet and gain of factors necessary for oocyte binding, leading to sperm capacitation (James et al., 2020). However, in birds, the role of sperm maturation in the epididymis remains unclear. The epididymis is much shorter in birds compared to mammals and sperm transits through the epididymis faster due to the high rate of sperm production. Indeed, spermatogenesis is a much longer process in mammals compare to birds as it ranges from 35 days in the mice to 74 days in man (Bennett, 1977). Furthermore, artificial insemination with testicular sperm does produce fertile eggs in poultry which suggests that maturation in the epididymis is not a necessary

condition for ovum fertilization (Munro, 1938; Howarth, 1983). However, other work has suggested that maturational changes do occur in birds as well. For instance, increasing sperm motility and velocity through epididymal transit has been reported in the domestic fowl (Munro, 1938; Ahammad et al., 2011a) and in the Japanese quail (Clulow and Jones, 1982). Similarly, in poultry, viability and fertilization capacity gradually increase from testes to vas deferentia (Ahammad et al., 2011a,b). The ejaculate is not only made of sperm cells. Indeed, after ejaculation spermatozoa are transported by seminal plasma, a very complex fluid composed of a diversity of molecules. Therefore, sperm cells interact with seminal proteins and other molecules impacting sperm physiology and fertilization outcome (Druart and de Graaf, 2018). Proteomics studies of seminal plasma in avian species have identified proteins associated with fertility in the turkey (Słowińska et al., 2017), in the chicken (Labas et al., 2015) and also with age and sperm velocity in the Red Junglefowl (Borziak et al., 2016). More recently, extracellular vesicles were found in chicken seminal plasma with different size and proteins markers between fertile and sub fertile males (Cordeiro et al., 2021). Another promising topic is the differences in miRNAs in high- and low-quality semen samples, miRNA patterns can be different depending on the male status and environmental changes, and differences between high and low motility samples were recently described in roosters (Xing et al., 2022). Further work comparing the composition of seminal plasma and the presence of different patterns of miRNAs of ejaculates between different storage time and between old and young males in the houbara bustard would be of great interest.

As for organismal aging, the rate of sperm aging is certainly not constant neither among-species nor among-individuals within species. The rate of sperm aging can be modulated by several factors (see Reinhardt, 2007 for a review). However, investment into mechanisms that limit age-associated damage does not come without cost. Therefore, natural selection could have shaped the evolution of anti-aging mechanisms according to potential fitness costs of accumulating old sperm in the ejaculate. In several bird species, females mate several times with the same male. Repeated copulations with the same male might provide freshly produced sperm for ovum fertilization. Actually, in some social species, females have been observed to eject sperm when mating occurs too early with respect to egg laying (White et al., 2008), which might represent a female strategy to avoid fertilization with old sperm. Spontaneous sperm discharge has also been suggested in males of several bird species, as shown by the presence of sperm in the cloaca of individuals sampled during the spring migration (before the mating season had started) (Quay, 1985). Sperm release might be a mechanism allowing males to get rid of sperm that had been stored for long time before reaching the breeding ground. The North African houbara bustard is a solitary bird dwelling at very low density in rocky deserts and steppes (Monnier-Corbel et al., 2022). Although males cluster in leks during the mating season, the lek sites are dispersed over much larger areas compared to classical lekking species. Therefore, the mating frequency of males is certainly very low and probably most of them only mate a handful of times (or maybe never) during the whole mating period. In species where males have reduced sexual opportunities, it is straightforward to predict the evolution of mechanisms allowing to protect stored sperm from oxidative attack. This might also apply to sperm that are stored in the female sperm storage tubules which preserve the sperm viability for weeks.

Our experimental set up involved homospermic (single male) inseminations. Accordingly, our measure of reproductive success only refers to the male capacity to fertilize eggs that hatch and produce viable offspring. Obviously, a different picture might emerge when ejaculates of different males compete for ovum fertilization. Female houbara bustards do mate with multiple males during the same reproductive bout, therefore there is scope for sperm competition to operate in this species (Lesobre et al., 2010). Under sperm competition, selection on traits promoting ovum fertilization is exacerbated (Vuarin et al., 2019b). For instance, small reduction in motility might have no consequences under homospermic mating, whereas sperm with lower motility can be easily outcompeted if females mate with multiple males. Previous work conducted on the houbara bustard has shown that when sperm of young and old males compete for ovum fertilization (during mixed sperm inseminations), young males have a substantial advantage since they have a larger paternity share compared to the 1:1 null hypothesis (Vuarin et al., 2019a). Therefore, an extension of the present work could consider performing competitive inseminations using sperm of different ages. Future work should also extend the period of sperm storage to fully assess the possible duration of sperm lifespan and perhaps increase the likelihood to observe sperm aging in this species.

## Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

## Ethics statement

Ethical review and approval was not required for the animal study because the breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate Independent Veterinarian (mandate number: 534–98), providing control of the wellbeing of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled.

## Author contributions

YH, MS-J, LL, GS, and JT conceived the study. LM, NR, and JT collected the data. HAH analyzed the data and prepared the tables and figures. YH and LL curated and supervised the conservation breeding program. LM and GS wrote the first draft of the manuscript. All authors edited, revised, and approved the final version.

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## Conflict of interest

LM, HAH, YH, LL, and JT were employed by Reneco International Wildlife Consultants LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.977184/full#supplementary-material

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Jean-Francois Lemaitre,
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REVIEWED BY
Csongor Vágási,
Babeş-Bolyai University, Romania
Victor Ronget,
Université de Lyon, France

*CORRESPONDENCE
Milan Vrtílek
vrtilek@ivb.cz
Martin Reichard
reichard@ivb.cz

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# Evidence for reproductive senescence across ray-finned fishes: A review

Milan Vrtílek[1]*, Jakub Žák[1,2] and Martin Reichard[1,2,3]*

[1]Institute of Vertebrate Biology, Czech Academy of Sciences, Brno, Czechia, [2]Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czechia, [3]Department of Ecology and Vertebrate Zoology, University of Łódź, Łódź, Poland

The origin, incidence, and consequences of reproductive senescence vary greatly across the tree of life. In vertebrates, research on reproductive senescence has been mainly focused on mammals and birds, demonstrating that its variation is largely linked to critical life history traits, such as growth patterns, juvenile, and adult mortality, and reproductive strategy. Fishes represent half of the vertebrate taxonomic diversity and display remarkable variation in life history. Based on a thorough literature review, we summarize current evidence on reproductive senescence in ray-finned fishes (Actinopterygii). While survival and physiological senescence are acknowledged in fish, their potential age-related reproductive decline has often been disregarded due to the prevalence of indeterminate growth. We demonstrate that age-related reproductive decline is reported across fish phylogeny, environments, and traits. An important point of our review is that the incidence of reproductive senescence in a species depends on both the number of studies for that species and the coverage of its maximum lifespan by the study. Reproductive senescence was documented for one-third of the studied fish species, with females suffering an age-related decline in reproductive traits less often than males or both parents combined. Neither parental care nor migratory strategy corresponded with the occurrence of reproductive senescence in fish. The traits that were affected by reproductive senescence most often were sex-specific, with pre-mating and mating categories of traits declining in females and sperm quality and quantity in males. We also demonstrate that reproductive senescence can be buffered by indeterminate growth. We provide rich evidence of reproductive senescence across ray-finned fishes, but we highlight the need for better data on age-related reproduction in fishes.

KEYWORDS

**reproductive aging, fish, Actinopterygii, indeterminate growth, lifespan, fecundity, life-history evolution, negligible senescence**

## 1 Introduction

Individual survival and offspring recruitment are two fundamental parameters of population dynamics. It is therefore important to understand age-related changes in mortality and in reproductive success, especially in exploited populations and populations facing environmental changes (Venturelli et al., 2012). At the individual level, it is the physiological decline that affects the prospect of survival and the potential decrease in the quality and quantity of offspring, which leads to senescence (Shefferson et al., 2017). The onset and rate of reproductive senescence clearly affect individual fitness (Bouwhuis et al., 2012; Austad and Finch, 2022), although, they are traditionally considered secondary aspects of overall senescence (Comfort, 1954). The organismal diversity in the rate of actuarial (demographic) senescence received particular attention (Jones et al., 2014), but a robust framework for specific analyses of the pace and shape of reproductive parameters has only recently been developed (Baudisch and Stott, 2019). The most comprehensive data on organismal age-related reproductive decline have so far come from avian and mammalian studies (Ricklefs et al., 2003; Lemaître and Gaillard, 2017; Lemaître et al., 2020; Vágási et al., 2021). The understanding of reproductive senescence across major clades of vertebrates is largely incomplete (Finch and Holmes, 2010; Nussey et al., 2013; Jones et al., 2014; Ivimey-Cook and Moorad, 2020), and existing gaps prevent the identification of potential ecological and evolutionary covariates and factors associated with reproductive senescence. This is a pressing problem as some lineages show a markedly higher rate of senescence than others (Ricklefs, 2010).

In this study, we address reproductive senescence in fishes, a group characterized by indeterminate growth and fecundity tightly related to body size (similar to non-avian reptiles; Hoekstra et al., 2020). Fishes are therefore an attractive target to search for evolutionary correlates of negligible senescence (Vaupel et al., 2004; Roper et al., 2021). As older fish become even larger, their reproductive output increases (Woodhead, 1998; Reznick et al., 2002), often disproportionately to their body size (Barneche et al., 2018). Indeed, the importance of large old females is well recognized in fish-stock assessments (Green, 2008). Ray-finned fishes (Actinopterygii) represent half of all vertebrate species (34,800 out of 70,000; IUCN, 2021; Froese and Pauly, 2022), which is reflected in their incredible life history and ecological diversity (Helfman et al., 2009). Fishes exhibit almost any mode of reproduction expressed across vertebrates (Wootton and Smith, 2015). Post-reproductive lifespan, typical for certain mammalian species (Cohen, 2004), is unexpectedly also reported in live-bearing fishes (Poeciliidae; Krumholz, 1948; Reznick et al., 2006). On the other hand, Pacific salmons (*Oncorhynchus* spp.; Crespi and Teo, 2002; Morbey et al., 2005) are an emblematic group known for a "big bang" reproduction, semelparity. The semelparous fish reproduce only once in a lifetime after which they die, and they are not restricted to salmonids (e.g., a galaxiid *Galaxiella pusilla*; Pen et al., 1993). Fishes contain both one of the earliest reproducing (*Nothobranchius furzeri*) and the longest reproductively active vertebrate species (*Sebastes aleutianus;* Vrtílek et al., 2018; Kolora et al., 2021). Overall, ray-finned fishes offer an opportunity to study multiple perspectives of life-history evolution and reproductive senescence, in particular.

Reproduction imposes a physiological cost that intensifies with age [hypothesized by Orton (1929) in Kelley (1962) and empirically studied by Craig (1977)]. This leads to a lowered allocation to reproduction, including skipping spawning in some years, to mitigate the costs that might increase the risk of mortality. Several studies on captive individuals indicated that reproductive senescence is prevalent in fish (Krumholz, 1948; Rasquin and Hafter, 1951; Woodhead, 1974a,b). The pathophysiological changes are in many aspects similar to mammals (Rasquin and Hafter, 1951; Patnaik et al., 1994). Reproductive senescence may express through various traits linked to offspring production, from mating behavior through gonad malfunction to offspring viability (Lemaître and Gaillard, 2017; Monaghan and Metcalfe, 2019). Our current knowledge of reproductive senescence from fish is, however, limited and fragmented (Finch and Holmes, 2010). The existing reviews on fish senescence (Craig, 1985; Patnaik et al., 1994; Woodhead, 1998; Reznick et al., 2002) are already outdated as the evidence that fish experience reproductive senescence is accumulating both from captive (Gasparini et al., 2019; Žák and Reichard, 2021) and wild populations (Karjalainen et al., 2016; Benoît et al., 2018). In this review, we aim to investigate published literature records for data on reproductive senescence across fish species with the overarching question "What evidence do we currently have for an age-related reproductive decline in ray-finned fishes?" We further explored three hypotheses on the occurrence of senescence in fish.

While fish were often considered immortal (thanks to their indeterminate growth) in the first half of the 20th century (Bidder, 1932), Comfort (1961), and Woodhead (1974a,b) demonstrated senescence in small ornamental fish. Given our growing understanding of the effect of indeterminate growth on reproductive senescence across vertebrates (Reinke et al., 2022), we hypothesized that, with more recent research, we will observe an increase in the proportion of studies reporting reproductive senescence in fishes.

Migration requires additional resources and increases the risks, and migratory birds and mammals show a faster pace of life than non-migratory species (Soriano-Redondo et al., 2020). We predicted that migrating strategy of a population will incur a higher probability of finding senescence in fish reproductive traits.

In addition, we consider an association between parental care and the occurrence of reproductive senescence. Parental care extends contact between the offspring and its parent(s)

and exerts significant costs on the caring parent (Clutton-Brock et al., 1989). It, therefore, has the potential to increase the negative impacts of senescence on the next generation. We predicted that species providing parental care will therefore be more likely to show reproductive senescence.

## 2 Methods

The present literature review is focused on examining evidence for reproductive senescence in fish. We defined three selection criteria for assessing an article to be suitable for our review. The article should be on ray-finned fish (Actinopterygii), which implies that we should exclude studies on sharks, rays, lampreys, etc. The second criteria was that the age of the fish should be determined by reading growth rings from otoliths, scales, fin rays, or bones, or through a known identity of the individuals (e.g., by marking or rearing them in captivity). We, therefore, excluded studies that assigned age using population body size distribution, for example, because variation in individual growth trajectory may bias correct age identification. The last criterion was that the study should look at a reproductive trait in relationship with the age of the fish. Overall, we aimed at an expanded selection of articles, including those that do not directly focus on reproductive senescence, to obtain a wider perspective on the relationship of reproduction with age and body size across fishes. A reproductive trait was any trait associated with offspring production, that is, related to mating ability, gonads, egg, or sperm amount and quality, fertilization, number and performance of the offspring, and parental care (see below for details). Only studies on adult fish were thus included in our database for review. We preregistered the review at Open Science Forum.[1]

On 28 September 2021, we performed two searches for literature on fish reproductive senescence, one on the Web of Science (WOS)[2] and the other on Scopus.[3] We used string representing our three criteria related to reproduction, age effect, and fish:

(reproduc* OR fertil* OR fecund* OR egg* OR breed* OR "offspring" OR hatch* OR parent*) AND (senesc* OR "ageing" OR "aging" OR "age" OR old*) AND (fish OR teleost* OR actinopteryg* OR pisc*).

We obtained 12,617 and 16,685 results from WOS and Scopus, respectively (29,302 in total). We downloaded bibliographical data for the articles, including the title and abstract, and merged the two datasets into a single xls file. We screened the collected articles in two steps: to identify suitable studies and then to extract relevant data for age-related reproductive traits from these studies.

In the first step, we screened titles and abstracts of the articles and only kept those that were on Actinopterygii and measured the effect of age on some reproductive traits. The excluded studies were labeled as "off-topic" (not on fish biology), "not age" (age not properly determined or on subadult stages), or "repage missing" (age was recorded but no reproductive trait was followed). For the original collection of articles, see the doi: 10.6084/m9.figshare.20200337. We provide an overview of the screening steps and the number of articles in a PRISMA flowchart (Moher et al., 2009), as given in **Supplementary File 1**.

We performed a second, thorough search in full-texts of the pre-selected articles (1,249 after duplicates were removed), particularly their Sections "Methods" and "Results." At this stage, reviews were searched for an additional primary source of data (we screened 17 additional primary articles detected in reviews, which made the total number of articles 29,319). We were not able to recover the full text for six articles, and these were eventually removed from the final database. In the second screening, we aimed to confirm that our three selection criteria were indeed met and we extracted study-, species-, and trait-specific information from each article. In addition to the already obtained bibliographical data, we searched for details on the study environment and setup, species, age, and sex of the fish, and most importantly for information on the reproductive trait and its relationship with age and body size. Full annotation of our final database is available in **Supplementary File 2**, so we provide only a brief overview here.

The environmental factors extracted were climate (category according to general climate zones–tropical, subtropical, temperate, or polar) and type of aquatic habitat (freshwater, brackish, or marine). We noted the study setup as whether it was captive or wild and whether authors targeted specific age classes (targeted study) or investigated the available age distribution (observational study). We assigned the currently accepted Latin name to the study species (according to FishBase, Froese and Pauly, 2022) with its taxonomic position (according to Fish Tree of Life, Rabosky et al., 2018). We also searched for species with the maximum known lifespan and mode of parental care (viviparous, biparental, maternal or paternal guarding, and no care or unknown) in FishBase (Froese and Pauly, 2022). We then noted using the age-determination method from the study, the minimum and maximum age of the fish, their sex (female, male, or both for parental traits), whether they were sampled alive or dead, and whether the study population was migratory or not. For each record of reproductive trait, we included its general description and grouped traits into trait types given the reproduction sequence with "pre-mating and mating," "gamete quality," "gamete number," "gonads," "pre-hatching," or "post-hatching" categories. We assessed the direction of the age effect in relation to reproductive success (i.e., positive or negative age effect rather than positive or negative correlations), whether age was continuous or categorical variable, what test was used, and

1 https://osf.io/m5rwa
2 www.webofscience.com
3 www.scopus.com

whether the effect size was measured. We similarly proceeded to the relationship between the reproductive trait and body size. In addition to that, we noted body size variable (e.g., somatic weight or standard length) and its goodness-of-fit relative to age (typically $r$ or $R^2$). At the end, we had to drop one article where the effect of age on reproductive traits was tested, but the direction was unknown. The final dataset can be found by following the doi: 10.6084/m9.figshare.20200337.

We identified indications of reproductive senescence based on a negative or bell-shaped relationship between age and a reproductive trait. Not all studies statistically tested the relationship between age and reproductive traits. Some contained only verbal statements about the relationship. In others, we had to infer the direction from mean values for age classes or plotted data. The direction of the relationship between age and a reproductive trait was therefore based on our interpretation of the study results, be it a statistical test, figure, or a plain verbal claim. We interpret the evidence for reproductive senescence across a combination of parameters mainly using pivot tables. We summarize the proportion of the records with reproductive senescence together with the overall number of records of the age–reproduction relationship. The general patterns are similar between studies from the subset containing statistically analyzed data and the entire collection. We, therefore, present the results from all studies collected. For the results with only the records that were statistically tested in the primary literature, refer to **Supplementary File 3**.

We tested the effect of publication year on the occurrence of reproductive senescence in a study-specific manner. All studies that contained at least one record of a negative or bell-shaped relationship between a reproductive trait and age were taken as positive cases (1, reproductive senescence present) and those without any evidence for negative or bell-shaped relationship were scored as negative (0). For species, we tested the effect of research attention and the presence of parental care on the probability of reporting reproductive senescence in a similar manner. Reproductive senescence in a species was a binomial response variable (0, if no study found reproductive senescence in that species or 1 for species with a record of reproductive senescence). The number of studies per species or the presence of parental care ("yes" for viviparity or parental guarding and "no" for no care, species with unavailable information were removed) were the explanatory variables. We tested the effect of the publication year, the effect of the number of studies in a species, and the species' parental care on finding reproductive senescence using three different binomial generalized linear models (GLM).

We further analyzed the coverage of species-specific lifespan by maximum age in studies from our database. For each record, we calculated the lifespan index as a ratio between the study's maximum age and the documented species' lifespan from FishBase (Froese and Pauly, 2022). We then tested the effect of the study setup (captive vs. wild) on lifespan index distribution using a $t$-test. In addition to that, we tested the relationship between lifespan index and the occurrence of reproductive senescence using a binomial generalized mixed-effects model (GLMM) from package 'lme4' v.1.1-27 by Bates et al. (2015). Reproductive senescence in a record was a response variable (coded 0 or 1 as above), lifespan index was an explanatory variable, and we included study ID as a random effect (there were multiple records from some studies). Statistical analyses were performed in R v.4.0.5 (R Development Core Team, 2021).

# 3 Results

## 3.1 Overview of records of fish age-reproduction relationships

The initial search yielded 29,319 articles. After screening and removing the duplicates, 409 studies conformed with our three-selection criteria, namely being ray-finned fish (Actinopterygii), measuring age, and recording at least one reproductive trait. We extracted 1,078 individual records of reproductive traits related to age across 258 fish species belonging to at least 35 taxonomic orders (**Figure 1**). The species with the most commonly retrieved relationship between age and a reproductive trait were rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), and vendace (*Coregonus albula*), all with approximately 4% share of the entire dataset. The most represented taxonomic orders were Cypriniformes, Salmoniformes, and Perciformes with 24, 16, and 16% shares, respectively.

With respect to the environment, records on age-related reproduction came mostly from temperate freshwaters (38%), followed by subtropical freshwaters (22%), and temperate marine environments (17%) (**Table 1**). Our database of reproductive parameters demonstrated a strong sex-biased distribution in data on the relationships between age and a reproductive trait (i.e., irrespective of a reproductive senescence pattern). Most (74%) of the age-related reproductive traits were measured in females, 20% were from males, and 5% were from both sexes. Most records (65%) were from wild populations and 35% were obtained in captivity. The most-recorded reproductive trait was female gamete quantity (34%), followed by female gamete quality (14%) (**Table 2**).

## 3.2 Evidence for reproductive senescence

A negative or bell-shaped relationship between age and a reproductive trait was considered to be a sign of reproductive senescence. In general, we found reproductive senescence in

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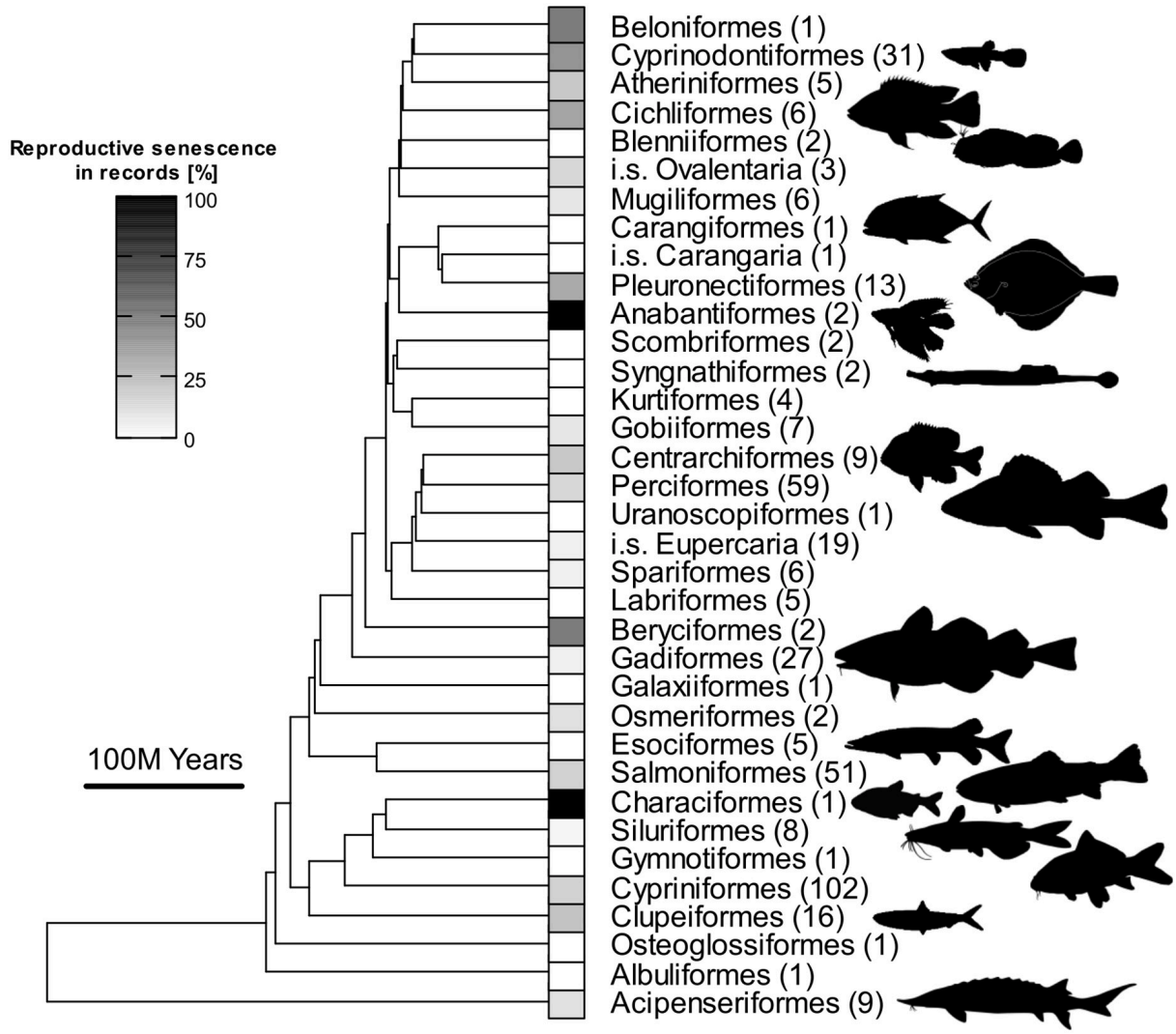


FIGURE 1
Records of reproductive senescence across the phylogeny of ray-finned fish (Actinopterygii). More intense black at the tip square points illustrates a higher proportion of negative or bell-shaped relationships between a reproductive trait and age among all the age-reproduction relationships (proportion of reproductive senescence records). The numbers in brackets show the total number of studies for each fish order. Fish silhouettes come from phylopic.org and fishualize R package (https://CRAN.R-project.org/package=fishualize). Please note that only the orders with a record of age effect on reproduction are presented.

26% of the suitable studies (106 of 409 studies) and a total of 19% of age-reproductive trait records (205 of all 1,078 records). In the section below, we detail individual factors that might have an effect on the incidence of reproductive senescence in fish.

### 3.2.1 The year when the study was published

During the 20th century, the existence of reproductive senescence in fish gradually gained recognition. We tested whether the number of articles reporting reproductive senescence increased over time. However, the effect of publication year on the proportion of studies with a record of reproductive senescence was not apparent (binomial GLM: $df = 407$, $z = 1.01$, $P = 0.313$).

### 3.2.2 Study environment and age manipulation

The type of climate and the aquatic environment were not associated with the incidence of reproductive senescence, with approximately 20% of the records in each category (Table 1). The only exception was tropical freshwaters, where the incidence of reproductive senescence was particularly high (56%) (Table 1). This is most likely due to the tropical freshwater species, such as *D. rerio*, *Poecilia reticulata,* or *N. furzeri,* being often studied specifically for senescence in the laboratory.

TABLE 1 Distribution of the reproductive senescence records across different climatic and aquatic environments.

| Climate | Aquatic environment | | | |
|---|---|---|---|---|
| | Freshwater | Brackish | Marine | Total |
| Polar | – | – | 0.18 (22) | 0.18 (22) |
| Temperate | 0.15 (409) | 0.17 (18) | 0.13 (178) | 0.15 (605) |
| Subtropical | 0.19 (237) | 0.00 (1) | 0.10 (110) | 0.16 (348) |
| Tropical | 0.56 (98) | – | 0.20 (5) | 0.54 (103) |
| Total | 0.22 (744) | 0.16 (19) | 0.13 (315) | 0.19 (1,078) |

The decimal numbers provide the proportion of negative or bell-shaped relationships between a reproductive trait and age (reproductive senescence). The numbers in brackets show the total number of records for each combination (all age-reproduction relationships).

TABLE 2 Senescence in different reproductive traits separated for male and female fish.

| Trait type | Female | Male | Both | Total |
|---|---|---|---|---|
| Pre- and mating | 0.51 (41) | 0.23 (47) | – | 0.39 (88) |
| Gonads | 0.21 (112) | 0.29 (62) | – | 0.24 (175) |
| Gamete number | 0.12 (355) | 0.39 (33) | – | 0.14 (388) |
| Gamete quality | 0.11 (154) | 0.41 (46) | – | 0.18 (201) |
| Pre-hatching | 0.24 (50) | 0.24 (21) | 0.32 (28) | 0.26 (99) |
| Post-hatching | 0.05 (87) | 0.09 (11) | 0.34 (29) | 0.12 (127) |
| Total | 0.15 (801) | 0.30 (220) | 0.33 (57) | 0.19 (1,078) |

The decimal numbers provide the proportion of negative or bell-shaped relationship between a reproductive trait and age (reproductive senescence records) and the numbers in brackets show the total number of records (all age-reproduction relationships) in that category.

The records from captive studies were more likely to report reproductive senescence than studies from the wild populations (31 vs. 13%). This was more important for the separation of the records than the contrast between observational and targeted studies. A total of 26% of the records from targeted studies (that manipulated age distribution) demonstrated reproductive senescence, while 16% of age-reproductive trait records showed signs of senescence in observational studies. This suggests that wild fish populations are much less likely to exhibit measurable reproductive senescence compared to captive populations, without much influence of age distribution manipulation.

### 3.2.3 Migration

We predicted that migratory strategy is more likely to be associated with a negative age effect on reproductive performance than non-migratory. Only 16% of the records of reproductive traits, however, showed a decline with age in migratory populations compared with 20% in non-migratory populations.

### 3.2.4 Parental care

The presence of viviparity or parental guarding did not explain variation in the occurrence of reproductive senescence among fish species (binomial GLM: $df$ = 242, $z$ = 0.52, $P$ = 0.601). The mode of parental care (viviparity, parental guarding, or no care) does not increase the proportion of senescence in records related to fish mating or post-fertilization traits (pre- or post-hatching) in either sex (**Supplementary File 4**). The same pattern was apparent for gamete quality, quantity, and gonad-related traits (data not shown).

### 3.2.5 Type of reproductive trait

In females, the highest proportion of records with reproductive senescence belongs to the "pre-mating and mating" category (51%), such as breeding frequency or proportion of reproductively active females. In males, the quality and quantity of sperm are the main traits indicating reproductive senescence (41 and 39%, respectively). Where the age of both parents was recorded, both pre- and post-hatching categories of offspring traits demonstrated a relatively high proportion of senescence effects as well (i.e., one-third of the records, refer to **Table 2**). Senescence thus seems to affect different types of reproductive traits between sexes.

### 3.2.6 Maximum age coverage (lifespan index)

To analyze species-specific lifespan coverage by the maximum age in our database, we collected data on species lifespan for 745 of all age-reproduction records (69%). The lifespan index (ratio between the study's maximum age and species' lifespan) was higher in studies of wild populations compared to captive studies (study-specific $t$-test: $t_{57}$ = 2.70, $P$ = 0.009; **Figure 2A**). Records with higher lifespan indexes were more likely to report reproductive senescence (binomial GLMM test: $N$ = 745, $z$ = 2.04, $P$ = 0.042; **Figure 2B**). This means that records from the wild populations and with reproductive senescence were collected from individuals of older age (closer to the known species' maximum age).

### 3.2.7 Body size confounding the effect of age

Older fish individuals are also larger due to their indeterminate growth. This may bias the analysis of the age effect on reproductive traits if this relationship is not properly accounted for (e.g., with ANCOVA). As the ANCOVA and models combining age and size were relatively uncommon in the collected studies (158 records, 15% of all), we decided to directly compare the effects of these two variables in the records of reproductive traits. The effect of body size on a reproductive trait was measured along with age for 527 records (49% of our dataset). In 48 records of reproductive senescence, 31% of body size effects were negative or bell-shaped. For the records without reproductive senescence (479), 96% had either no or a positive effect of body size on the reproductive trait.

In addition to this, we retrieved goodness-of-fit measures of both age and body size for 304 records. Only 21 of those were for records of reproductive senescence, where the body size variable was a superior fit compared to the age for 16 records of

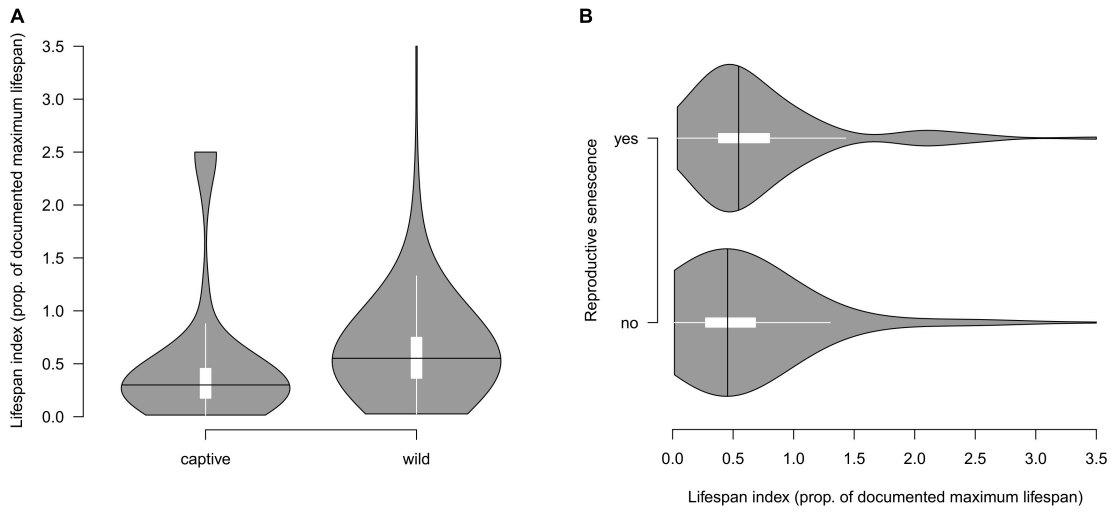


FIGURE 2
The coverage of species-specific maximum lifespans. The lifespan index is a ratio between the study's maximum age and documented species' lifespan. **(A)** Distribution of lifespan index in records from captive and wild populations. **(B)** Relationship between lifespan index and confirmed reproductive senescence. The width of the violin plots illustrates the density of the data points, the white inset box represents the interquartile range and the black crossing line indicates the median of the lifespan index. Note that the age reported in some studies exceeded the maximum known age for the species (retrieved from FishBase, Froese and Pauly, 2022).

reproductive traits (76%) and an inferior fit for 5 records (24%). For the records without clear signs of reproductive senescence, body size was generally (74%) a better fit to the reproductive traits data than age. Overall, the two subset analyses suggest that body size has indeed often a stronger explanatory power in fish reproductive trait variation than the age effect.

## 3.3 Species focus

We found signs of reproductive senescence in at least one study for 31% of species from our article collection. Relatively, more studied species had a higher probability to provide evidence of reproductive senescence because the likelihood to find a record of reproductive senescence for the particular species increased with the number of studies (binomial GLM: $df = 256$, $z = 3.87$, $P < 0.001$).

### 3.3.1 Frequent species

In the four most frequently studied species (together 16% of all records of age-reproductive trait relationships), reproductive senescence appeared more often (30%) than in the remaining, less commonly studied species (17%; the overall average being 19%). Among these four most-represented species, there was no consistent pattern in reproductive senescence in the trait categories that yielded at least 5 age-reproduction records. The reproductive traits that showed sufficient representation were mainly related to female ovaries, egg number, and egg quality (together 47% of all age–reproduction records in the four species). Rainbow trout (*O. mykiss*) females showed reproductive senescence of 20% in gamete number and 14% in post-hatching records, but none in the gamete quality. Vendace (*C. albula*) females exhibited reproductive senescence of 40% in gonad-related traits, 38% in egg number, and 19% in egg quality records. In contrast, females of common carp (*C. carpio*) did not show any signs of reproductive senescence in gonad-related traits, egg number, or egg quality. Zebrafish (*D. rerio*) was represented mainly by male traits and showed a larger prevalence of reproductive senescence with 83% in pre-mating and mating, 57% in sperm number, 67% in sperm quality, and 29% in pre-hatching traits records. The pre-hatching traits (such as fertilization rate or embryo survival) were also recorded for both parents in zebrafish and they showed a higher incidence of reproductive senescence compared to males-only records (50% of the pre-hatching trait records).

# 4 Discussion

We assembled studies providing evidence for reproductive senescence in fishes across various phylogenetic lineages, contexts, and traits. The collected records span from demographic aspects of reproduction in wild populations (Carter et al., 2014; Benoît et al., 2018) to laboratory studies directly focused on reproductive senescence (Woodhead, 1974a; Žák and Reichard, 2021). Different fish species represent a particularly diverse evolutionary clade that includes some exceptionally long-lived species. They can thus provide insights into the evolution of aging (Kolora et al., 2021) and perhaps contribute to the understanding of how to curb its negative

effects on our own species (Woodhead, 1998). Being a long-lived species does not necessarily imply escaping senescence, at least not at the physiological or reproductive level. Although there are indeed examples of long-lived fish species reproducing relatively more successfully when growing older (Berkeley et al., 2004), our review demonstrates this is not a universal rule across ray-finned fish.

## 4.1 Comparative context of reproductive senescence in ray-finned fish

We found that reproductive senescence in fish is taxonomically widespread. We considered either a bell-shaped or a negative relationship with age as a sign of reproductive senescence. More than 30% of the fish species exhibited some form of reproductive senescence, and the variation across the phylogeny was large. Senescence has been recorded in all age-related reproductive traits observed in labyrinth fishes [Anabantiformes, two studies by Woodhead, 1974a,b] and was also relatively common in tooth carps (Cyprinodontiformes). On the other hand, the frequency of reproductive senescence was very low in cods (Gadiformes) and catfishes (Siluriformes), despite them being well-represented in the collection of age-related reproductive traits. Within taxonomic orders, the variation was also high. In Cypriniformes, zebrafish display a high incidence of reproductive senescence, while reproductive senescence was reported at a very low frequency in the common carp. Across fish species, parental care, and migratory strategy did not increase the occurrence of reproductive senescence.

In mammals or birds, reproductive senescence is much more prevalent. Similar reviews focusing on female reproduction found that 68% of mammalian and 61% of avian species exhibited some form of age-related decline (Lemaître et al., 2020; Vágási et al., 2021). Although there are rare studies on reproductive senescence from reptile females (Hoekstra et al., 2020), a broader overview of the occurrence of reproductive senescence across vertebrates is not available. The lower prevalence of reproductive senescence in fish compared to mammals or birds is not surprising given the indeterminate growth and body size-dependent fecundity in fish. Mammalian females invest relatively more energy in reproduction for an extended period of time compared to a typical female fish. Mammalian offspring depend completely on milk provision from their mothers with lactation being the most energy-demanding phase of raising the offspring (Clutton-Brock et al., 1989; Speakman, 2008). The high allocation that mammals and birds put into parental care likely increases the potential for age-related reproductive decline, and behavioral and bodily deterioration also reduces their ability to gather sufficient nutrition toward older ages. The parental care hypothesis was not, however, supported by our analysis. We did not find a relationship between fish viviparity or parental guarding and the occurrence of reproductive senescence. Another important aspect of mammalian reproduction is that females possess a fixed pool of oocytes that deteriorate throughout their lives, unlike fish species which produce new eggs continuously (Lubzens et al., 2010). This may also increase the likelihood of a reproductive decline in mammals compared to fish. We would like to emphasize that reproductive senescence is much more widespread across fish than generally assumed, and we found cases of reproductive senescence in species that serve as textbook examples of non-senescent lineages (e.g., *Sebastes alutus*, de Bruin et al., 2004). In addition to that, the absence of reproductive senescence in certain lineages may stem from data deficiency rather than being a genuine aspect of their life history, and we elaborate on that in the below section.

## 4.2 Sex-related aspect of fish reproductive senescence

The distribution of records on age-related reproductive traits was markedly skewed toward females in our database. It is a common problem across studies on age-related reproduction in vertebrates that data from males are under-represented (Nussey et al., 2013; Lemaître and Gaillard, 2017; Archer et al., 2022). Reproductive senescence was, however, more frequently recorded for males or both parents combined rather than for females (30 and 33% compared to 15%). This is perhaps because most female-related studies focused on fecundity (**Table 2**), a trait tightly associated with body size in fish (Wootton and Smith, 2015). Female fish usually employ different life-history strategies compared to males. For example, females mature later and at a larger size, they grow slower but for a longer period of time, and they live longer than males (Woodhead, 1998; Wootton and Smith, 2015). The sexes also differ in their strategy of energy allocation into reproduction (Wootton and Smith, 2015). It is, therefore, reasonable to expect sex-specific differences in age-related reproductive decline as reported, for example, in red deer (*Cervus elaphus*), where males suffer from a steeper decline in annual fecundity than females (Nussey et al., 2009).

Female and male reproductive senescence also differed in the most frequently affected traits, a finding corroborating previous data across vertebrates (Tompkins and Anderson, 2019). In females, pre-mating and mating traits, such as inter-brood interval or regular reproduction, were most prone to age-related decline. This is likely a consequence of energetic or physiological limitations toward the end of life, where females reduce the number of reproductive events (instead of, for example, the number of eggs). Inter-brood interval extends in older mammalian females as well (see Karniski et al., 2018). While females may generally prefer to lay more of smaller eggs (Einum and Fleming, 2000), the selection should favor offspring

quality over quantity with increasing female age (Moorad and Nussey, 2016). Both egg-related traits (quantity and quality) exhibited very low occurrence of reproductive senescence in our database. While they did not analyze any data for fish, Ivimey-Cook and Moorad (2020) found a negative effect of maternal age on offspring survival in wild mammals, but positive in wild birds. Our analyses do not provide any support for the role of parental care in reproductive senescence, as neither viviparity nor guarding of the offspring was associated with the increase in its incidence.

In male fish, the amount and quality of sperm declined with age more often than other reproductive traits. Sperm-related senescence also occurs in birds (sperm motility: Møller et al., 2009; DNA damage: Velando et al., 2011; sperm number: Cornwallis et al., 2014) and some mammals (Lemaître and Gaillard, 2017). These patterns can be surprising because the amount and quality of sperm directly affect male reproductive success (Stoltz and Neff, 2006). Although some general patterns for senescence in the types of reproductive traits emerged from our database, the high diversity of traits (more than 260 different reproductive traits collected) prevented us from performing a thorough quantitative analysis of the collected records on age-related reproduction.

## 4.3 Reproductive senescence in free-living and captive fish

Studies that were designed in captivity showed a markedly higher proportion of reproductive senescence records compared with those performed on fish populations in the wild. There is no overall concordance across animal studies comparing senescence between captivity and the wild. Some taxa, such as mammals or squamates, survive better in captivity (Scharf et al., 2015; Tidière et al., 2016) and also have lower rates of senescence (Lemaître et al., 2013), while others, such as turtles or antler flies, show mixed results (Kawasaki et al., 2008; da Silva et al., 2022). Captive mammal males often show signs of age-related decline in sperm traits (Lemaître and Gaillard, 2017), but comparative data from the wild are very rare (but see Curren et al., 2013). In fish, studies on age-related reproduction from the wild had unexpectedly better coverage of species-specific maximum age than studies from captivity. This may be because some of the captive studies were performed on commercial stocks, where keeping individuals for an extended period (until reproductive decline) is unlikely desirable (Green, 2008). A captive setting, on the other hand, offers better control over multiple factors compared to research conducted in the field. Captive stocks are also protected from natural stressors that may result in the selective disappearance of senescent individuals from the population. Assuming a strong link between physiological and reproductive senescence, as reported in birds and mammals (Ricklefs et al., 2003), senescent individuals are more likely to succumb to predation or disease in the wild (Martin and Festa-Bianchet, 2011; Bouwhuis et al., 2012; Hämäläinen et al., 2014). This makes them considerably under-represented in older age cohorts, and selective disappearance consequently masks the negative relationship between physiological or reproductive conditions and age at the population level (Maklakov et al., 2015). A higher survival rate and ability to track individual fish thus probably increased the probability of captive studies to record reproductive decline as the fish aged.

## 4.4 Under-represented old cohorts

It is obvious that some fish species maintain high reproductive performance until advanced age (at least compared to human lifespan), but may display signs of reproductive senescence. The fecundity of the oldest (48 years old) examined giant grenadiers (*Albatrossia pectoralis*) was superior to younger females (Rodgveller et al., 2010). Similarly to that, females of rougheye rockfish (*S. aleutianus*) possess fully functional ovaries with no signs of atresia until the age of at least 80 years. Their ovary mass, however, declines at an advanced age (de Bruin et al., 2004). Among shorter-lived species, captive male platyfish (*Xiphophorus maculatus*) kept their reproductive potential and the levels of reproductive hormones to the oldest ages recorded (5 years), but high fibrosis in their testes was apparent (Schreibman et al., 1983). This suggests that focusing on a single trait may ignore the age-related decline in others and underappreciate reproductive senescence. According to our database, there is no record of reproductive senescence for 70% of the studied fish species. The patchiness of evidence for reproductive senescence may have arisen from the lack of high-quality demographic data. In particular, poor coverage of maximum lifespan in age-reproduction investigations is obvious for fishes. Few studies from our database actually sampled fish populations toward the maximum known lifespan. The studies considerably under-represented older age cohorts because three-quarters of the records were from individuals that did not even reach 75% of the known species' maximum lifespan. This especially applies to long-lived species, such as the giant grenadier or the rougheye rockfish mentioned above with a maximum lifespan of 56 and 205 years, respectively (data on maximum age comes from Froese and Pauly, 2022). Across the studies from our database, a higher lifespan index (ratio between the study's maximum age and species' known lifespan) was associated with a higher likelihood of recording reproductive senescence. Sampling toward the maximum lifespan is a natural requirement for the proper estimation of the impact of reproductive senescence. This is, however, complicated for the long-lived fish species by data availability in the first place and the ability to accurately determine their age in the wild (Craig, 1985). The insufficiency of high-quality demographic data thus

prevents us from making a more robust general conclusion about reproductive senescence in ray-finned fish.

### 4.5 Role of indeterminate growth in fish reproductive senescence

Ray-finned fish species are characterized by continuous growth throughout their lives (Bidder, 1932; Charnov et al., 2001). This makes them candidates for negligible and negative senescence because large (and hence old) individuals are generally favored by natural selection (Bidder, 1932; Vaupel et al., 2004; Jones and Vaupel, 2017). This is because mortality (actuarial senescence) increases as a function of age but declines with body size (Caswell and Salguero-Gómez, 2013; Colchero and Schaible, 2014; Jones and Vaupel, 2017). In fact, body size explained variations in fish reproductive traits better than age in 75% of cases in our database. On the other hand, for records with an age-related reproductive decline in a trait, only one-third of the body size effects were negative or bell-shaped. This may be a consequence of variation in growth strategy across fish species. For example, turquoise killifish (*N. furzeri*) or Trinidadian guppy (*P. reticulata*) reaches body size limits long after maturity, and reproductive senescence occurs after cessation of their growth (Reznick et al., 2006; Žák and Reichard, 2021). Indeterminately growing fish, such as cods (Gadiformes) (Woodhead, 1998), demonstrate a very low incidence of reproductive senescence. This suggests that the negligible senescence can indeed be characteristic of some fish lineages. In reptiles, for example, it often arises that even after body size correction, the reproductive trait does not decline with age (Warner et al., 2016; Tully et al., 2020; but see Sparkman et al., 2007). Among indeterminately growing invertebrates, common woodlouse (*Armadillidium vulgare*) females of older ages also produce a higher number of larger offspring. Yet, the offspring of older female woodlouse have lower fitness, offsetting the increase in maternal fertility arising from the indeterminate growth (Depeux et al., 2020). The current datasets indicate that indeterminate growth itself is not sufficient to escape reproductive senescence. To provide any solid conclusion on the effect of indeterminate growth on reproductive senescence, we need that future studies on age-related reproduction properly account for the confounding effect of body size, that is, by using body size as a covariate.

### 4.6 Conclusion

Reproductive senescence is present and diverse across ray-finned fish species. The indeterminate growth may indeed, to some extent, buffer against the age-related reproductive decline, but growing larger is often not sufficient. The understanding of the evolution of fish reproductive senescence would definitely benefit from more empirical life-history studies with the effort put into covering the species' lifespan and accounting for the size-related fecundity. It is important to state that our review inevitably suffers from attention bias. The studies focused on one or a few traits, and the set of focal traits varied notably among studies. The overall insight into what actually occurs during the aging of particular species is therefore still incomplete. Interspersed records of age-related reproductive decline across the fish phylogeny and the identified sampling bias indicate that reproductive senescence is prevalent in fishes. The present overview of the up-to-date published records thus offers a new, more elaborate perspective on fish life history, and we hope that our review will stimulate further research in the most neglected directions.

## Author contributions

MV: conceptualization (equal), data curation (lead), formal analysis (lead), investigation (lead), methodology (lead), visualization (lead), writing – original draft (lead), and writing – review and editing (equal). JŽ: conceptualization (equal), data curation (supporting), formal analysis (supporting), investigation (equal), methodology (supporting), writing – original draft (supporting), and writing – review and editing (equal). MR: conceptualization (equal), funding acquisition (lead), investigation (supporting), methodology (supporting), resources (lead), supervision (lead), writing – original draft (supporting), and writing – review and editing (equal). All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.982915/full#supplementary-material

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EDITED BY
Jean-Michel Gaillard,
Université Claude-Bernard Lyon 1, France

REVIEWED BY
Fernando Colchero,
University of Southern Denmark, Denmark
Samuel Pavard,
Muséum National d'Histoire Naturelle, France

*CORRESPONDENCE
Ronald Lee
✉ rlee@demog.berkeley.edu

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# Theoretical perspectives on reproductive aging

Ronald Lee[1]* and C. Y. Cyrus Chu[2]

[1]Graduate School in Demography and Economics, University of California, Berkeley, Berkeley, CA, United States, [2]Institute of Economics, Academia Sinica, Taipei, Taiwan

Age patterns of female reproduction vary widely among iteroparous animal species with determinate growth. Often fertility declines with age, but in other cases, it may be flat or rise across age. Sometimes fertility ceases altogether, leaving a substantial post-reproductive life span. In this article, we discuss theories that may provide some insights into how these diverse patterns might evolve. We present a simple optimal life history model and consider circumstances in which fertility might rise or fall with age. In our model, without assuming that costs per birth rise with age, that foraging efficiency declines, or that net intergenerational transfers increase, we find that optimal fertility would tend to rise rather than decline. This happens because less energy would be allocated to survival at older ages, leaving more to allocate to fertility. In our analysis, optimal fertility could decline at older ages when an older female makes heavy net intergenerational transfers to multiple offspring or grandoffspring, reducing resources for her own reproduction. This pattern is more likely to evolve when costs of fertility at older ages are higher, when costs of reducing juvenile mortality are low, and when the level of juvenile mortality is high. We also derive conditions for the evolution of menopause, for determinate growth, and for juvenile mortality that declines with age. The optimal life history can exhibit a variety of age patterns of fertility, rising, flat, or falling, depending on the constraints and opportunities faced.

KEYWORDS

**optimal life history, reproductive senescence, reproductive aging, intergenerational transfers, fertility, reproductive decline, fertility theory, life history theory**

## 1. Introduction

Why do humans and other animals age and die? This question has attracted much theoretical and empirical attention in recent decades. The question of why fertility declines with age in so many species has attracted far less attention (Monaghan et al., 2008).

Empirical studies have found a wide variety of age patterns of fertility across the animal kingdom. For most but not all mammals fertility declines at older ages

(Jones et al., 2014; Lemaître et al., 2020), although in some species, it is flat or rises with age. Non-mammalian vertebrates often have flat or rising fertility with age. Birds have slow or negligible reproductive aging (Holmes et al., 2003). Reptiles, tortoises, and amphibians often have slow or negligible aging (da Silva et al., 2022; Reinke et al., 2022). Invertebrates show all patterns, but most often fertility declines at older ages (Jones et al., 2014). A number of species besides humans and toothed whales have substantial post-reproductive survival, including nematodes and rotifers (Jones et al., 2014).

A theory of reproductive aging must be sufficiently flexible to accommodate these many different possible age patterns. Here, we explore how and why evolution might sometimes lead to reproductive senescence, but at other times to flat or rising fertility and at still other times to menopause or lifetime infertility. Fertility is centrally important in evolutionary theory, but we suggest that it cannot be analyzed in isolation from other key aspects of life history such as survival, growth, foraging efficiency, parental and grandparental transfers, and broader social organization. For this reason, we begin with a review of some topics in life history theory and their relation to fertility.

Medawar (1952/1957) and Williams (1957) initiated modern evolutionary thinking on senescence and death. Hamilton (1966) formalized one of their central insights, that deleterious mutations that raised mortality at older ages would reduce reproductive fitness less than those raising it at younger ages, and so natural selection would remove them from the population more weakly and slowly at older ages, causing mortality to rise with age. This approach, emphasizing mutation accumulation, has been developed further (Charlesworth, 1994; Caswell and Salguero-Gómez, 2013; Wachter et al., 2014). According to this approach, senescence is "purely maladaptive" (Partridge and Barton, 1993) and occurs because the forces of selection are not sufficiently powerful at older ages to rid the population of continuously occurring deleterious mutations.

If applied to fertility in the same way, the mutation accumulation approach implies that fertility should decline with age in proportion to declining survivorship as measured by the life table $l_x$ function (Hamilton, 1966). However, Hamilton (1966) warned us that this approach would be less useful for fertility: "...it is not so plausible that a gene could simply add an element of fertility at a given age without affecting the rest of the schedule as it is that a gene might cause the elimination of a single element of mortality." Raising survival by eliminating a deleterious mutation would not necessarily require additional energy. However, adding a birth would certainly require nutritional energy taken either from siblings or from the mother's own bodily reserves, which would adversely affect fertility and survival at other ages. A mutation accumulation approach can still be adopted but these energetic tradeoffs would need to be taken into account and would make the theory more complicated (Lee, 2003).

A very different approach develops the idea that senescence is adaptive. If an organism was to allocate more energy to maintaining its soma and postponing death, it would require energy that could otherwise be used for reproduction earlier in life, perhaps reducing fitness. This is the disposable soma theory of Kirkwood (1977) which has been developed in formal models of optimal life history theory. In these models, an organism acquires energy at each age and allocates it among somatic maintenance and survival, fertility, and growth. Under natural selection, the organism moves toward the pattern of energy allocation by age which maximizes its lifetime reproductive fitness as beneficial mutations are positively selected rather than through the negative selection of deleterious mutations, as emphasized by Hamilton.

Mutation accumulation theory cannot explain or predict age patterns of fertility and mortality *de novo*. Its predictions about mortality depend on a given age pattern of fertility and conversely. Optimal life history theory does provide an initial age pattern of rates (based on many assumptions), but it ignores the insights of mutation accumulation theory. A full account would require attention to both. Partridge and Barton (1993) provided worked-out examples and a useful diagram that illustrates the interaction of the two evolutionary processes. Mutation accumulation would blur and modify the optimal life history results, but the basic shapes of the age schedules would reflect the optimal patterns. Danko et al. (2012) concluded that mutation accumulation could raise old age mortality but would have very little effect on the evolved mean age at reproductive maturity, which was very largely determined by adaptive (optimizing) forces. These considerations suggest the value of an approach that can derive the evolutionarily optimal age profiles themselves that reflect tradeoffs in energetic allocations across the life history.

Others have applied the optimal life history approach to age patterns of fertility when there are tradeoffs with mortality and fertility at later ages, a topic called "the general life history problem" (Charlesworth and Leon, 1976; Stearns, 1992, 2000; Charlesworth, 1994). The main result, as summarized by Stearns (2000), is that when mortality is higher at a range of adult ages, optimal reproduction will be higher before those ages and lower after those ages.

Our approach is related to the general life history problem, but we impose more structure. We develop a simple optimal life history model to consider the joint evolution of age patterns of fertility, mortality, growth, and intergenerational transfers. We use variations on our basic optimal life history model to investigate interrelated topics relevant to reproductive senescence. Tradeoffs between fertility and mortality occur through the energy budget equation at each age and intergenerational transfers are also explicitly taken into account. This formulation makes the results easier to interpret (we hope) but also reduces their generality.

We now briefly introduce the main topics or scenarios that we will consider.

## 1.1. Menopause

Hamilton (1966), of course, realized that extensive post-reproductive survival in women was inconsistent with his analysis of senescence, but he noted "As remarked by Williams, an obvious excuse for this discrepancy is to be found in the factor of parental care," a point closely related to the "Grandmother Hypothesis" (Hawkes et al., 1998), and the thrust of our model and analysis here. While we realize there are many theories of the evolution of menopause, for example, as reviewed in a game theory framework by Thouzeau and Raymond (2017), we believe that intergenerational transfers are central. The budget constraint of an isolated individual is constrained by the energy it acquires itself at each age through foraging, which it then allocates at that age among growth, survival, and reproduction. However, in many species, females (and sometimes males) make significant investments in their offspring after birth, e.g., feeding, guarding, sheltering, training, or warming them. When such investments extend over time, perhaps leading to multiple simultaneously dependent offspring (as with humans), then later life fertility could be affected. The extreme case here is menopause with lengthy post-reproductive survival. We analyze the conditions under which this menopause pattern would be optimal. Due to the costs of supporting multiple dependent offspring, this typically occurs in a broader social context in which grandmothers and other kin, and possibly non-kin as well, help invest in the offspring. A study of the Tsimane, a contemporary Amazon Basin forager-horticulturalist group, illustrates this point (Hooper et al., 2015:S3). Average total net caloric transfers to children and grandchildren are nearly as high at ages in the 50s as in the 30s and 40s and they remain substantial through the 60 s before declining. Distant kin and non-kin also make transfers. Another recent study draws on data from multiple hunter-gatherers and horticulturalist groups to estimate the direct fitness from own fertility and indirect fitness from transfers, taking relatedness into account. It concludes that "Under reasonable assumptions, these [indirect fitness] benefits are the equivalent to having up to several more offspring after age 50." (Davison and Gurven, 2022, text in square brackets added).

## 1.2. The age pattern of juvenile mortality

Hamilton (1966) wrote "None of the schedules of forces of selection . . . can account for the rise in mortality in younger and younger pre-reproductive ages." Indeed his analysis implies that mortality at all ages before reproductive maturity should be constant and very low because death at any pre-reproductive age entails a loss of 100% of reproductive fitness, whether the age is younger or older. This predicted low and flat mortality pattern is inconsistent with observed juvenile mortality as Hamilton was well aware, paralleling the problem with human post-reproductive survival. Again, he suggests that ". . .parental care brings the necessary trend," although he did not include it in his model. As we incorporate intergenerational transfers in our model, we can show that juvenile mortality should instead decline with age (Lee, 2003; Chu et al., 2008), here demonstrated in a new way. However, we recognize that these transfers can be only one among a number of reasons why infant/juvenile mortality declines with age since this also occurs in species that do not make transfers after birth.

## 1.3. Determinate growth vs. indeterminate growth with lifelong reproduction

An earlier study (Taylor et al., 1974) showed that with a linear budget constraint, the optimal life history would initially invest solely in survival and growth and then, after a certain age, switch to investing solely in survival and fertility, a pattern known as "determinate growth." With a non-linear budget constraint, however, the optimal outcome could instead be "indeterminate growth" in which resources are allocated to growth, survival, and fertility throughout the entire life span. This can result in "negative senescence" in the sense that body size grows over a life span leading to declining mortality and increasing fertility (Vaupel et al., 2004; Baudisch, 2008). Mammals (with some possible exceptions) and birds exhibit determinate growth, while many fish, amphibians, and reptiles exhibit indeterminate growth. The distinction is very important since reproductive senescence is not to be expected in species with indeterminate growth. In this study, we will derive the determinate growth result in a new way.

## 1.4. Reproductive senescence in determinate growth species

We address the conditions under which evolved fertility may rise, fall, or be flat in determinate growth iteroparous species like mammals and birds. Our analysis, which builds on the preceding topics, includes the roles of intergenerational transfers, foraging efficiency, and the energetic costs of fertility and survival. We believe that our analysis and results here are new. Perhaps, most strikingly, we find that if none of these just-listed factors varies with age (e.g., if net transfers do not rise with age and if foraging efficiency does not decline), then optimal fertility would rise with age, the opposite of reproductive senescence. Later, we will

discuss the interpretation of this result and suggest an alternative approach for future research.

## 2. Formal analysis of reproductive aging in optimal life histories

We focus on female fertility in iteroparous animal species with determinate growth. Our models are generally single sex. For simplicity of exposition, our models have at most three age classes that are sufficient to see whether fertility rises or declines with age and to derive other useful results. Readers interested in similar treatments with many age classes or with continuous age distributions will find them in our other articles such as Chu et al. (2008) and Chu and Lee (2013). Our modeling approach is conceptual in the sense that we do not try to derive realistic age schedules of fertility and mortality in contrast, for example, to the explicit results in Drenos and Kirkwood (2005) (who assume parametric fertility schedules that decline with age). Instead, our goal is to see under what conditions evolutionary forces might lead to different qualitative age patterns of fertility: decreasing or increasing with age or ceasing altogether as with menopause.

### 2.1. Optimal life history models

We consider several different one-sex (female) models in which each individual lives either one, two, or at most three periods. Everyone dies after period three. We call these periods age-0, age-1, and age-2. We interpret them as childhood, young adulthood, and old age, or sometimes as childhood, youth (Juvenilehood), and adulthood. We start with the most general version of the model, before considering special cases.

An individual starts life at age-0 with an initial body mass, $w_0$, provided by its mother out of her energy budget based on her evolved life history. With a given body mass $w_a$ at age $a$, an individual can acquire energy $\gamma w_a$ through foraging or hunting, where $\gamma$ is a coefficient expressing the way foraging efficiency (energy generation) is related to body size. If the foraging/hunting activity is carried out cooperatively with other co-residing kin members, the setting is different, and we will consider this case elsewhere. At each age $x \in \{0, 1, 2\}$, the individual may acquire energy both through foraging ($\gamma w_x$) and through a net transfer of energy from older individuals aged $a > x$. $T_a$ is the possible net energy transfer received or given at age $a$; $T_a > 0$ if one is receiving net energy at age $a$, and $T_a > 0$ if one is transferring net energy to others at age $a$.

We use the three-age scenario because it is the simplest structure needed to explain some age patterns of fertility and mortality. With an appropriate interpretation, this three-age setting is, in fact, quite general. If there are more than three ages, conventional dynamic programming says that individuals can always first optimize from age-3 onward, and then do the second-stage optimization by taking the optimized fitness value at the end of period 3 as given. Thus, age-3 can be thought of as including "the remaining life periods from age-3 onward."

In some cases to be discussed, we do not need three ages to tell the story, and then we will simplify the model accordingly. Sometimes we will need a minor modification to illustrate a point, and then we will introduce this modification only for that illustration.

Let us begin with an organism that after its birth must rely entirely on the energy it acquires on its own. At each age $a$, the organism acquires energy, $\gamma w_a$, and allocates it either to maintenance or reproduction. Reproduction requires energy to build and maintain the necessary organs, to produce eggs and provide nutrients, to find and acquire a breeding site, and to find and choose a mate (in more complicated two-sex models). Maintenance requires energy to avoid and repair somatic damage and avoid illness and predation raising the probability of survival to the end of the period. Specifically, for each individual, we have the following energy constraint:

$$b_a p_a + c_a m_a + d_a z_a = \gamma w_a \quad a = 0, 1 \tag{1}$$

where $b_a$ and $c_a$ are cost coefficients, $p_a$ is the probability the agent survives to the end of period $a$, and $m_a$ is the number of live births successfully produced.[1] The interpretation of Eq. 1 is that at age $a$, in order to increase $p_a$ (probability of surviving) by $\triangle p_a$, it needs $b_a \triangle p_a$ of energy input, and similarly for fertility $m_a$. The cost of providing the initial body mass for each offspring is included in the individual's cost per birth, $c_a$.

In this 3-age setting, the optimal life history in the evolutionary sense can be viewed as maximizing expected lifetime births,

$$max \left\{ l_0 m_0 + l_1 m_1 + l_2 m_2 \right\} \tag{2}$$

where $l_x = \prod_{i=0}^{x} p_i$. For any given $w_a$, $a = 0, 1, 2$, the control variables are $\mathrm{p_a}$ and $\mathrm{m_a}$. Therefore, the problem is to choose $\mathrm{p_a}$ and $\mathrm{m_a}$ to maximize $\{l_0 m_0 + l_1 m_1 + l_2 m_2\}$. Let an * indicate the optimal value, so the optimal controls are written as $\mathrm{l_a^*}$, $\mathrm{p_a^*}$, and $m_a^*$.[2]

Now, we consider possible transfers. Given any transfers $T_a$, $a = 0, 1, 2$, the available energy on the right-hand side of Eq. 1 becomes $E_a = \gamma w_a + T_a$. The same maximization can be

1 In a discrete-time setting, we must be careful about interpreting the order of events. Energy is a "flow" (measured for a period of time), whereas survival and reproduction are measured at the period's end, which corresponds to a stock (measured at a point in time). Different specifications of the order of events may involve slight changes in the formulation.

2 The more general form of the optimal life history (see Chu et al., 2008) is $\lambda^3 = max_{p_a, m_a}\{\lambda^2 l_0 m_0 + \lambda l_1 m_1 + l_2 m_2\}$, where $\lambda$ is the implicit population growth rate. In stationary equilibrium, $\lambda$ would be driven to 1 (zero net growth), so that the problem is equivalent to solving Eq. 2. In what follows, we adopt the stationary scenario implicitly, and treat Eq. 2 as the objective function.

applied, and let us write the maximum as,

$$\pi(T_0, T_1, T_2) \equiv l_0^* m_0^* + l_1^* m_1^* + l_2^* m_2^* \quad (3)$$

If transfers are also to be chosen, evolution will drive the life history to choose the optimal transfers ($T_0$, $T_1$, $T_2$), subject to some additional constraints.

The most common transfers are between kin. In our one-sex, 3-age, stationary population setting, the equilibrium age structure evaluated at the period's end must be proportional to the following factors: $l_0$ for age-0, $l_1$ for age-1, and $l_2$ for age-2. This age distribution can be interpreted either as averages for generations of a given kin group or as averages for the population as a whole. The sum of net transfers given within the kin group or within the population must equal the sum of net transfers received, so net transfers must satisfy the following constraint:

$$l_0 T_0 + l_1 T_1 + l_2 T_2 = 0 \quad (4)$$

Thus, the optimal transfer problem is to maximize $\pi(T_0, T_1, T_2)$ in Eq. 3, subject to the constraint in Eq. 4 (for a fuller discussion of this constraint and its interpretation at the family level, refer to Lee, 2008).

To summarize, we separate the optimal life history exercise into two steps, namely, the first step is to maximize over $\{p_a, m_a\}$ for any given transfers $(T_0, T_1, T_2)$ and the second step is to choose the optimal $T_a'$s.

Now, we consider some specific scenarios.

## 2.2. Scenario 1: Menopause

We interpret menopause as an optimal corner solution (i.e., at the zero-fertility lower bound) of old-age fertility and will explain when and why this unique optimality may arise in our simple model. To focus on the discussion of $m_2$, we consider the scenario $m_0^* = 0$ and $T_0 > 0$, $T_1 < 0$, and $T_2 < 0$, meaning that the age-0 needs energy transfers from older ages and is not sexually mature. We skip the conditions that generate these scenarios for the time being[3] and focus the discussion on the age-3's (grandmother's) decisions.

If the optimal solution involves $p_2^* > 0$ and $m_2^* = 0$, then it says that it is optimal for the grandmother to survive but not reproduce. Intuitively, this is more likely to happen when $\gamma w_2$ is large (the grandmother is more capable of foraging, perhaps due to her accumulated knowledge) so that she produces a lot of energy, and $c_2$ is large so that it is costly for the grandmother to reproduce, so some of her acquired energy may be transferred out. However, what exactly are the mathematical conditions for $p_2^* > 0$, $m_2^* = 0$ to happen?

Given $m_0^* = 0$, the objective function becomes,

$$\pi \equiv \max\left\{p_0 p_1 m_1 + p_0 p_1 p_2 m_2\right\}$$

We consider a small perturbation in the grandmother's net transfers such that $|T_2|$ becomes marginally larger, with the increased transfers all going to age-0, and correspondingly $m_2$ becomes marginally smaller, with all other control variables held unchanged. Since $m_0 = 0$, the increased transfer to age-0 would only increase $p_0$. Thus, the change in fitness is,

$$d\pi = p_1 m_1 dp_0 + (p_1 p_2 m_2)\, dp_0 + (p_0 p_1 p_2)\, dm_2 \text{ or}$$

$$\frac{d\pi}{dm_2} = p_1 m_1 \frac{dp_0}{dm_2} + (p_1 p_2 m_2)\frac{dp_0}{dm_2} + p_0 p_1 p_2$$

The energy saved by a fertility change of $dm_2$ at age-2 is $-c_2 dm_2$. Referring to the equilibrium population age distribution earlier, we see that the proportion of individuals at age-2 divided by the proportion of individuals at age-0 is $p_1 p_2$. This implies that each individual at age-0 would receive $-p_1 p_2 c_2 dm_2$ energy, that is, gain energy if $dm_2$ is negative and fertility at age-2 declines. Therefore, age-0 would increase her survival probability by,[4]

$$\frac{dp_0}{dm_2} = \frac{-p_1 p_2 c_2}{b_0}$$

Thus,

$$\frac{d\pi}{dm_2} = -p_1 m_1 \frac{p_1 p_2 c_2}{b_0} (-p_1 p_2 m_2)\frac{p_1 p_2 c_2}{b_0} + p_0 p_1 p_2$$

$$= -p_1 p_2 \left[p_1 m_1 \frac{c_2}{b_0} + (p_1 p_2 m_2)\frac{c_2}{b_0} - p_0\right]$$

$$= -\frac{p_1 p_2}{p_0}\left\{\frac{c_2}{b_0}\left[p_0 p_1 m_1 + p_0 p_1 p_2 m_2\right] - p_0^2\right\}.$$

Recall our assumption that at an optimum, the population is in a stationary equilibrium. The abovementioned expressions do not necessarily refer to an optimum, but if the life history moves slowly along an evolutionary trajectory, then the population is always near a stationary equilibrium which implies that $\pi$ (the net reproduction ratio) will be near 1.0. Note that the first two terms in the square brackets equal $\pi$. Using this, and substituting 1.0, we find,

$$\frac{d\pi}{dm_2} = -\frac{p_1 p_2}{p_0}\left\{\frac{c_2}{b_0} - p_0^2\right\} = \frac{p_1 p_2}{p_0}\left\{p_0^2 - \frac{c_2}{b_0}\right\}.$$

This derivative will continue to be negative as long as $p_0^2 < \frac{c_2}{b_0}$, and age-2 will continue to evolve in the direction

3 These conditions are not difficult to imagine: For instance, if $c_0$ is very large, then it is very costly for the age-0 to reproduce, therefore $m_0^* = 0$ must appear. And if $E_0 = \gamma w_0$ is very small, then the age-0 must rely on transfers from seniors to survive.

4 Alternatively, one can combine Eqs. 1 and 4 and use the assumption $m_0 = 0$ to obtain,

$$p_0 = \frac{\gamma w_0}{b_0} - \frac{p_1\left[T_1 + p_2\left(b_2 p_2 + c_2 m_2 - \gamma w_2\right)\right]}{b_0}.$$

Taking derivative yields $dp_0/dm_2$ in the text.

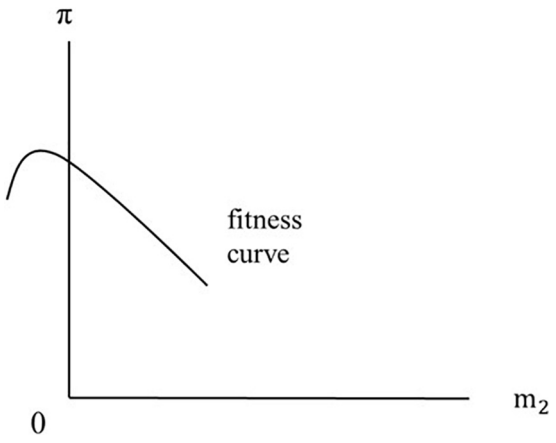


FIGURE 1
Menopause occurs when the fitness curve has a non-positive slope around the vertical axis. If the slope around the vertical axis is exactly zero, then $d\pi/dm_2 = 0$.

of reducing its fertility $m_2$ and transferring more to the age-0, gradually raising $p_0$ until finally an interior optimum is reached where $p_0 = \sqrt{\frac{c_2}{b_0}}$ or, alternatively, arrives at a corner due to one of two possible boundaries. One boundary occurs at $p_0 = 1$ when $\frac{c_2}{b_0} > 1$. Another possible boundary occurs if $m_2$ reaches zero.

While $p_0 = 1$ is a possible outcome in our model, we believe it has never been observed in nature so we will focus on the possible corner outcome for fertility. We consider the possibility of reaching the fertility boundary while $\frac{d\pi}{dm_2}$ is still negative, indicating that lower fertility, were it possible, would lead to greater fitness. From the result earlier, this is more likely to happen when $\frac{c_2}{b_0}$ is large, that is, the cost of raising fertility for an older female is substantially greater than the cost of reducing the mortality of a young offspring or grandoffspring. It is also more likely to happen when $p_0$ is low so that despite the transfers from older women as their fertility falls, the survival of young grandoffspring has remained low.

A positive value for $\frac{d\pi}{dm_2}|_{m_2=0,\ dm_2<0}$ tells us that fitness would be raised even more if it were possible for fertility at age-2 to become negative. Since this is not possible, $m_2 = 0$, a corner solution, will give the highest feasible fitness. If $d\pi/dm_2 = 0$ when $m_2 = 0$ that would indicate an interior solution optimum that just happened by chance to occur at the corner.

It is also important to note that a trajectory ending in menopause is possible only if the fertility of younger adult women can rise high enough to achieve a net reproduction ratio of 1. As older women increase their transfers to their grandoffspring, their daughters can reduce theirs and raise their fertility. We will return to this point later.

Menopause occurs when the fitness curve has a non-positive slope around the vertical axis. Figure 1 illustrates this situation, plotting the fitness index against $m_2$. As drawn, the fitness curve crosses the vertical axis (at $m_2 = 0$) with a negative slope, indicating that the optimum is a corner solution. This corresponds to the life-history interpretation of "menopause." If $d\pi/dm_2 = 0$ at $m_2 = 0$, then the slope of the fitness curve around the vertical axis is exactly zero.

Here, in our simple model, menopause evolves as the end point of declining fertility at older ages. However, in a more flexible model, the same forces at work could equally well apply to unchanged fertility at older ages together with the evolution of longer post-reproductive survival. Menopause is known to occur in humans and four species of toothed whale (Ellis et al., 2018). Transfers in human hunter-gatherer-horticulturalist groups have been extensively studied (Hooper et al., 2015; Davison and Gurven, 2022).

## 2.3. Scenario 2: The age pattern of mortality from birth to maturity

In this scenario, we interpret the three ages as childhood, youth, and adulthood. Suppose $c_0$ and $c_1$ are large, then the first two ages may not be appropriate for reproducing. In this case, age-2 is the age of sexual maturity. Or, using the alternative interpretation we discussed earlier (for additional age groups using dynamic programming), the individual is sexually mature from age-2 onward. We also assume that only the age-2 is strong enough to acquire sufficient energy to transfer to ages 0 and 1.

For species without parental transfers, Hamilton's theory predicts that the probability of surviving to the next age should be the same for age-0 and age-1 (in fact, for all ages before reproductive maturity). If there are parental transfers to children and youth, we will argue that mortality should instead be higher at the younger ages and survival lower than later in youth, that is, $p_0 < p_1$. This implies there is high childhood (infant) mortality followed by a decline. If there are more than three ages, then the survival probability will decline after the age of sexual maturity due to the usual senescence argument. Thus, $p_0 < p_1$ constitutes a sufficient condition for U-shape (in our 3-age setting, V-shape) mortality of species with parental transfers. In what follows, we derive conditions for this to happen.

Given $m_0 = m_1 = 0$, we have the following simplified objective function:

$$\pi \equiv \max \left\{p_0 p_1 p_2 m_2\right\}$$

Let the giving-end age-2 transfer to age-1 be $T_1'$ and to age-0 be $T_0'$. The energy received by an individual at age-1 is $p_2 T_1'$ and at age-0 is $p_1 p_2 T_0'$. Again, these are obtained by dividing the ratio of the proportion of individuals at age-2 by that at age-1. Thus, the corresponding survival probability is,

$$p_0 = \frac{\left(\gamma_0 w_0 + p_1 p_2 T_0'\right)}{b_0}$$

$$p_1 = \frac{\left(\gamma_1 w_1 + p_2 T_1^{'}\right)}{b_1}$$

Now suppose age-2 individuals change their strategy for transferring energy, reallocating energy from age-0 to age-1 ($dT_0' = -dT_1'$), holding all other controls unchanged. We can see the change in survival probability would be

$$dp_0 = \frac{p_1 p_2 dT_0^{'}}{b_0}$$

$$dp_1 = \frac{-p_2 dT_0^{'}}{b_1}$$

The change in the fitness index is,

$$\frac{d\pi}{dT_0^{'}} = \left(\frac{p_0 p_2}{b_1} - \frac{p_1^2 p_2}{b_0}\right) p_2 m_2$$

If we are at an optimum, then this perturbation must have zero effect on fitness, π, so,

$$\frac{p_1^2}{b_0} - \frac{p_0}{b_1} = 0$$

This equation implies that $p_1 = \sqrt{\frac{b_0 p_0}{b_1}}$.

If we make the neutral assumption that $b_0 = b_1$, that is that the energetic costs of improving survival in childhood and youth are equal, then we have $p_1 = \sqrt{p_0}$ in which case $p_1 > p_0$ since $p_0 < 1$. This means that mortality before adulthood declines with age, as, in fact, it generally does. Even if $b_0 < b_1$, there would still be a range of values of $p_0$ for which mortality would be declining with age.

This result says that if sexually mature adults transfer energy to offspring at more than one immature age, then it is always more efficient for the younger age to face a higher mortality rate than the older. The intuition of our result is that since our objective function is $p_0 p_1 p_2 m_2$ and there are costs $b_0$ and $b_1$ for raising $p_0$ and $p_1$, maximization of $p_0 p_1$ would always lead to $p_0 = p_1$ because costs would be the least when the two are equal. However, cross-age transfers introduce additional cost concerns, making a death at age-1 costlier than one at age-0, because a death at age-1 would also waste the earlier transfers to the offspring at age-0. This "accumulation effect" creates a product term on the cost side, which is the reason why we have $p_1$ as a square root of $p_0$. In more general cases with many ages before sexual maturity, this accumulation effect would be amplified for ages closer to zero and farther from the age of sexual maturity. Thus, unless there are specific assumptions about the $b_a$ coefficients, for species with transfers from adults-to-children, it is likely mortality declines with age and survival increases up to sexual maturity. As noted earlier, this cannot be the whole story since mortality also declines in species without intergenerational transfers.

## 2.4. Scenario 3: Determinate growth

The objective of this scenario is to explain why a determinate growth pattern occurs. Let us forget about the transfers for a moment and consider life with only two ages, namely, age-0 and age-1. The individual invests energy $z_a$ in growth, raising body size by $d_a z_a$. The objective function is to maximize,

$$p_0 m_0 + p_0 p_1 m_1.$$

We assume that the energy constraint is also linear in $z_a$:

$$b_a p_a + c_a m_a + d_a z_a = \gamma w_a \quad a = 0, 1$$

where $w_1 = w_0 + z_0$, characterizing the somatic growth occurring through investment at the previous age, $z_0$.

Energy production increases with body mass as described by γ (body mass may also be interpreted more broadly here to include investment in the brain or in the acquisition of knowledge). Evidently, age-1 would never invest in $z_1$ because it is the last period of life, so $z_1 = 0$. Given the above, we will have determinate growth if at age-0 $z_0$ and $m_0$ are not both positive, since determinate growth is characterized by growth without reproduction followed by reproduction without growth.

Consider a marginal change of $dm_0 < 0$. This saves the age-0 individual an amount $c_0 |dm_0|$ of energy, which can be used to create $dz_0 = c_0 |dm_0| / d_0$ of somatic growth. Then, the corresponding body growth can generate $\gamma dz_0 = \gamma c_0 |dm_0| / d_0$ energy at age-1. This energy will allow an additional $[\gamma c_0 |dm_0| / d_0] / c_1$ age-1 births. Is this marginal change worthwhile? We check it by investigating the sign of,[5]

$$d\pi = -p_0 dm_0 + p_0 p_1 \frac{\gamma c_0}{d_0 c_1} dm_0$$

$$\frac{d\pi}{dm_0} = p_0 \left(p_1 \frac{\gamma c_0}{d_0 c_1} - 1\right).$$

Note that $c_0$, $c_1$, γ, and $d_0$ are all constants. For any given $p_0$ and $p_1$, since the above expression is independent of the perturbation $dm_0$, $(d\pi / dm_0) = 0$ will hold only by accident. Thus, with a linear budget constraint, it is impossible to have indeterminate growth: $m_0$ should either increase with a corresponding decrease in $z_0$ (which would mean that an organism reproduced over the rest of its life without growing at all following birth), or decrease with a corresponding increase in $z_0$, until a constraint boundary is hit, which corresponds to determinate growth as it is observed in nature.

Indeterminate growth, with both growth and fertility occurring together from the start, could occur in this model only with a non-linear budget constraint in which the incremental

5 Note that in order to evaluate the tradeoff between age-0 fertility and somatic growth, we design a specific direction of perturbation. This makes the derivatives look cleaner.

(marginal) fitness gains from energy spent on fertility or growth were not constant, but rather declined as the energy devoted to each increased. It is certainly plausible that this would be the case. For example, Metcalf et al. (2003) comparatively analyzed monocarpic perennial plants to test life history theories, reporting that rapid growth and low mortality tradeoffs in small plants are consistent with the budget constraint approach. However, they also find that growth is a decreasing function of size which suggests a non-linear budget constraint. Non-linearity in somatic growth could also arise if more rapid growth is less efficient. For example, suppose at age-0 growth is related to investment by $w_1 = w_0 + \zeta(\delta)$ where $\zeta(\delta)$ is non-linear and $\zeta'(\delta) > 0, \zeta''(\delta) < 0$, with growth becoming less efficient at higher rates. Then, an interior solution is possible with both growth and reproduction occurring. Other possibilities will be discussed later.

## 2.5. Scenario 4: Reproductive senescence

In this scenario, we study when fertility will decline with age. We consider a 3-period lifetime with determinate growth. At age-0, the body grows, and age-1 and age-2 are mature ages. The mature individual has a somatic capital $w^*$, which is a constant for age-1 and age-2, but different ages have different efficiency parameters ($\gamma_a$, a = 1, 2) to generate energy. The reason behind different $\gamma_a's$ might be depreciation or wearing out; for example, the age-2 may have the same body size as age-1 but be less healthy or agile than age-1, so that $\gamma_1 > \gamma_2$ (Lemaître et al., 2020: p. 8). We assume that age-0 generates 0 energy, but that is just for simplicity and convenience; it need not be so.

Let the giving-end transfer from age $a$ (a = 1,2) to age-0 be $|T_a|$. The net energy left for the age-a adult to allocate is, therefore, $\gamma_a w^* |T_a| E_a$.

The total energy received at age-0 is $p_1|T_1| + p_1p_2|T_2|$. Using this, the corresponding survival probability for age-0 is $p_0 = (p_1|T_1| + p_1p_2|T_2|)/b_0$ (because age-0 is not reproductive, all her energy is used in maintenance). For any given $|T_1|, |T_2|$, and $p_0$, we concentrate on the fertility decision of the adult:

$$\max\ p_0\left[p_1m_1 + p_1p_2m_2\right]$$

subject to the constraints $b_ap_a + c_am_a = E_a$. The choice variables are $p_a$ and $m_a$. Dynamic programming tells us that we should first solve the age-2 maximization:

$$\text{Max} p_2m_2 \text{ subject to } b_2p_2 + c_2m_2 = E_2.$$

This generates $m_2^* = \frac{E_2}{2c_2}$ and $p_2^* = \frac{E_2}{2b_2}$. Then, we substitute the optimized value $p_2^*m_2^*$ back to the age-1 formulation and solve the following problem:

$$\text{Max}\left[p_1m_1 + p_1p_2^*m_2^*\right] \text{ subject to } b_1p_1 + c_1m_1 = E_1.$$

Straightforward maximization shows that the age-1 solution is

$$m_1^* = \frac{1}{2c_1}\left[E_1 - E_2^2\frac{c_1}{4b_2c_2}\right].$$

To see whether there is reproductive senescence, we look at the difference,

$$m_1^* - m_2^* = \frac{E_1}{2c_1} - \left[\frac{E_2}{2c_2} + \frac{E_2^2}{8b_2c_2}\right]. \quad (5)$$

Evidently, reproductive senescence ($m_1^* > m_2^*$) is more likely to occur if (1) $c_1$ is small or $c_2$ is larger (age-1 is more efficient in reproducing than age-2), or (2) $E_1$ is large or $E_2$ is small due to reduced foraging efficiency with aging (age-1 has more disposable energy than age-2), (3) $E_2$ is small because $|T_2|$ is large (age-2 is an important supporter of age-0), or (4) high cost (large $b_2$) of achieving higher old age survival.

Case (1) occurs if the reproductive system tends to deteriorate with age, for example, the declining quantity and quality of ova in mammals and birds, raising the cost of reproduction with age. This deterioration could perhaps be offset by increased spending on maintenance. However, while that might reduce $c_2 - c_1$, it would also add the energetic cost of maintenance to the total cost of reproducing at age-2. Case (2) occurs if the productive soma tends to deteriorate and lose efficiency, so that $\gamma_1 > \gamma_2$, as reviewed by Lemaître et al. (2020: p. 8) (for simplicity, we have not subscripted $\gamma$ in the previous model but the meaning here should be clear).

Therefore, this condition, large $c_2/b_0$, would underly the result in Scenario 4 which found that small $E_2$ brings reproductive senescence. As $T_2$ is endogenous, this argument explains what is likely to make it larger and $E_2$ smaller.

In case (3), an older female's energy available for reproduction is depleted by her large net transfers to younger descendants, $|T_2|$, as she relies more heavily on investment in indirect fitness by using energy transfers to raise the fitness of grandoffspring rather than to reproduce herself. These transfers are themselves evolving as we discussed in Scenario 1. The greater the ratio $\frac{c_2}{b_0}$, the greater will be the evolved transfers from $T_2$ to $T_0$ and the lower, therefore, will be $E_2$. The polar case is menopause or $m_2 = 0$.

Case (4) occurs when for any reason such as higher extrinsic mortality or smaller body size survival at older ages is more costly to attain, consistent with a result by Charlesworth and Leon (1976).

In any event, it is important to note that reproductive senescence does not necessarily appear in optimal life history. Suppose that there is no change with age in the cost of fertility $c_a$, or foraging efficiency $\gamma_a$, or net transfers $T_a$ so that available

energy is also the same at both ages. Setting all these equal in Eq. 5, we find that,

$$m_2^* - m_1^* = \frac{E_2^2}{8b_2c_2} > 0.$$

That is, in this case, optimal fertility would rise with age rather than decline, because at older ages, investment in survival brings less fitness payoff (particularly when $b_2$ is large), unlike investment in fertility. Optimal energy expenditures are shifted from maintenance to fertility, which rises relative to age-1. This would also be true under somewhat weaker assumptions. In addition, these same forces would be at work to generate this result in a model with more ages. This equation seems consistent with the slow or negligible reproductive aging in birds since older birds do not make transfers to grandoffspring and, therefore, retain more energy for their own reproduction, and their cost of survival ($b_2$) is low since they fly.

## 3. Discussion

We will consider our results in a broader context, starting with menopause. Should menopause be considered an example of extreme reproductive senescence? Some analysts see it resulting from the evolution of longer life while reproductive senescence remained unchanged. For example, Ellison and Ottinger (2014) wrote: "But the Chu and Lee (2006) hypotheses assume that the evolved characteristic in humans is an early termination of reproduction relative to its ancestral state, whereas it seems clear that the evolved characteristic is prevalent and extended post-reproductive life, not premature reproductive cessation." Since our models, including here in Scenario 1, assume a preexisting life span and then analyze the optimal level of fertility in predefined old age, their criticism appears well-founded. Furthermore, our verbal interpretations of results typically have asserted the evolutionary ordering of change suggested in this quote (Chu and Lee, 2013), which in retrospect is unfortunate. It is unfortunate because in the mathematical theory, the ordering of change is irrelevant, and all that matters is the relative lengths of dependent childhood, fertility, and life span, not which trait is fixed and which one changes. We view the system of intergenerational transfers, menopause, and post-reproductive life as coevolving along with the development of the brain and the long period of maturation and dependence of offspring as described in the theoretical framework of Hawkes et al. (1998) or Kaplan et al. (2000) (which are not very different for a single sex model). We view menopause as an adaptive part of an optimal life history strategy. Accepting Ellison and Ottinger's (2014) conclusion that age at reproductive senescence remained fixed while longer life evolved still requires an explanation of fitness benefits derived from this lengthening post-reproductive period.

Consider the circumstances we find to be conducive to the evolution of menopause. First is the high fertility of younger adult females. For humans, it is well-known that reproductive-age women have short birth intervals and high fertility relative to other great apes (Thompson and Sabb, in press), made possible by transfers from others (Kaplan et al., 2000). Second, the energetic cost of a birth to an older woman should be high and the cost of raising the survival of young offspring through transfers to them should be low. How costly would it be for increased fertility at older female ages to evolve? The mammalian and avian reproductive systems with a fixed supply of oocytes at birth might make it more difficult and costly to continue fertility to higher ages. Elephants and some whales maintain fertility to far higher ages than humans, so it would arguably be possible. Nonetheless, it seems plausible that continuing fertility to high ages would be more costly for mammals than for other non-avian species. The cost of raising infant/juvenile survival for mammals might be lower than for other species because of the ability to feed infants efficiently over an extended period through lactation. Grandparents could indirectly contribute to infant nutrition by helping to feed the mother. Among mammals, humans with pair bonding and a long history of food sharing in social groups might again be well-situated to improve the survival of post-lactation juveniles. This is all speculative, of course, but perhaps a case could be made that $c_2/b_0$ is generally high for mammals and within mammals is possibly higher for humans.

Regarding mortality decline within the juvenile period, we have already discussed the difficulties with Hamilton's demonstration that mortality should be constant until reproductive maturity, which he himself did not believe, although as a refutation of Fisher's argument based on reproductive value, Hamilton's analysis remains convincing. Once intergenerational transfers (or "parental care," in his words) are introduced, Hamilton's argument no longer holds. Now, the older the juvenile the more has been invested in her so the greater the fitness loss if she should die (a corresponding forward-looking story can also be told). This seems correct so far as it goes. One difficulty is that mortality also declines in species that do not make transfers following birth. Orzack and Levitis (in press) present a further critique.

As we showed, a linear budget constraint leads to determinate growth and is inconsistent with indeterminate growth. However, the condition is sufficient but not necessary; some non-linear budget constraints would also lead to determinate growth. Indeed, even if a budget constraint were locally linear, it is difficult to imagine that it could remain linear over the entire evolutionary range, even as upper or lower bounds on variables were approached. The insight from the model is a useful starting point but is not the end of the story. A little later we will suggest a different approach in which the budget constraint is inherently nonlinear.

As noted earlier, reproductive senescence in the optimal life history is far from inevitable. If we make the neutral assumption that the costs of survival and fertility and the efficiency of foraging for a given body weight as well as intergenerational

transfer behavior all remain unchanged in the adult life history, then fertility will rise with age rather than decline, for reasons already discussed. We certainly could assume a tendency to senesce in any one of the three fundamental parameters in our model (b, c, or γ) which could lead to fertility decline, but that leaves open the question of why there was an insufficient investment to maintain these capabilities in the face of wear and tear. More important, in our analysis, is the possibility that intergenerational transfer behavior evolves in such a way as to reduce fertility at older ages while boosting the survival (and in a full model, growth as well) of young offspring. This possibility is contained within our model as we have discussed earlier, and is more likely to evolve when the ratio of the cost of raising old age fertility to the cost of improving survival for young offspring is high and the level of early life survival is low. While menopause is one polar outcome, intermediate outcomes with interior solutions with reduced old age fertility are also possible.

We also have some thoughts about future studies for ourselves or others on this topic. As we noted at the beginning, wear-and-tear has rightly been rejected as an adequate explanation of aging and senescence, because an organism could offset it by allocating resources to maintenance, repair, and replacement. To quote Williams (1957), "It is true, of course, that some parts of organisms do literally wear out. Human teeth, for instance, show wear similar to that of any tool subjected to friction, but this wear is no more a part of senescence than is the wearing away of replaceable epidermal cells. The senescence of human teeth consists not of their wearing out but of their lack of replacement when worn out."

It is possible, however, that current analytic approaches to reproductive aging, including ours in this article, go too far in the direction of banishing wear and tear from consideration. In our specification, for example, a unit of energy expended on reproduction at age $a$ will produce $1/c_a$ births. Nothing here says that $c_a$, the energy cost of reproduction at age $a$, does not vary with age. However, unless there is some theoretical reason to expect $c$ to rise with age, we cannot very well assume it does without smuggling in by the backdoor the very thing we want to show may exist.

In this regard, it could be useful to distinguish the somatic reproductive machinery as one variable, call it $K$, from the current energy allocated to reproduction and fed into the reproductive machinery, call it $f$. Then, fertility at age $a$, $m_a$, is a function $m_a = g\left(K_a, f_a\right)$ for which a specific example would be $m_a = hK_a^{\alpha} f_a^{1-\alpha}$. What we call the reproductive machinery, $K$, tends to wear out over time at the rate δ and the organism can offset some or all of this decay by investing energy $i$, giving $K_a = (1-\delta) K_{a-1} + i_{a-1}$. An investment in $K$ at any age will also raise all future levels of $K$ at subsequent ages, other things equal. These future benefits of investment in $K$ will be large for a young organism with its whole future ahead of it, but small for an older organism that is closer to the end of life. For this reason, in the optimal life history, $K$ will be at a peak early in life but then will decline because it becomes less worthwhile to offset the wear-and-tear at older ages. As a result, for any given expenditure of energy on current reproduction $f_a$, the increase in the birthrate will be less at older ages than at younger because the machinery has become less efficient or, we might say, the energy cost per birth $c_a$ has risen with age. It rises not by assumption but due to the optimal investment strategy. While maintenance of reproductive organs affects all future reproductive ages, the future is shorter for older than for younger females, so the incentive to invest at older ages is also less. This reasoning is very similar to that for rising mortality. Now, in a symmetric way, we have the possibility of reproductive senescence. We speculate that a model of this sort could account for the different patterns of fertility that rise, fall, or hold steady across age, as observed in nature. We think it is a promising avenue for future research.

## 4. Conclusion

The balance of these and perhaps other forces will determine age patterns of fertility and reproductive senescence. The weights received by each of these forces will in turn depend on many other aspects of the life history and sociality of the species, and perhaps on the environment as well. There is no single theoretical insight that will clarify reproductive senescence in the way that insights of Medawar, Williams, and Hamilton seemed, for a time, to show that rising mortality in adulthood would be inevitable. Reproductive life histories are far too complex and variable.

## Data availability statement

This manuscript is theoretical and does not analyze data. There is one chart but it is mathematical and does not use data.

## Author contributions

CC contributed more to the mathematical modeling. RL contributed more to the plan, structure, and writing of the manuscript. Both authors contributed to all parts of the manuscript and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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EDITED BY
Jean-Francois Lemaitre,
UMR5558 Biométrie et Biologie Evolutive (LBBE), France

REVIEWED BY
Gerald S. Wilkinson,
University of Maryland, United States
Marianthi Tangili,
University of Groningen, Netherlands

*CORRESPONDENCE
Britta S. Meyer
✉ britta@bsmeyer.com

†These authors have contributed equally to this work and share senior authorship

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# Sex-specific changes in autosomal methylation rate in ageing common terns

Britta S. Meyer[1]*, Maria Moiron[2], Calvinna Caswara[3], William Chow[4], Olivier Fedrigo[5], Giulio Formenti[5], Bettina Haase[5], Kerstin Howe[4], Jacquelyn Mountcastle[5], Marcela Uliano-Silva[3,4,6], Jonathan Wood[4], Erich D. Jarvis[5,7], Miriam Liedvogel[1,2†] and Sandra Bouwhuis[2†]

[1]Behavioural Genomics, Max Planck Institute for Evolutionary Biology, Plön, Germany, [2]Institute of Avian Research, Wilhelmshaven, Germany, [3]Berlin Center for Genomics in Biodiversity Research, Berlin, Germany, [4]Wellcome Genome Campus, Wellcome Sanger Institute, Cambridge, United Kingdom, [5]The Rockefeller University, New York, NY, United States, [6]Department of Evolutionary Genetics, Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany, [7]Howard Hughes Medical Institute, Chevy Chase, MD, United States

Senescence, an age-related decline in survival and/or reproductive performance, occurs in species across the tree of life. Molecular mechanisms underlying this within-individual phenomenon are still largely unknown, but DNA methylation changes with age are among the candidates. Using a longitudinal approach, we investigated age-specific changes in autosomal methylation of common terns, relatively long-lived migratory seabirds known to show senescence. We collected blood at 1-, 3- and/or 4-year intervals, extracted DNA from the erythrocytes and estimated autosomal DNA methylation by mapping Reduced Representative Bisulfite Sequencing reads to a *de novo* assembled reference genome. We found autosomal methylation levels to decrease with age within females, but not males, and no evidence for selective (dis) appearance of birds of either sex in relation to their methylation level. Moreover, although we found positions in the genome to consistently vary in their methylation levels, individuals did not show such strong consistent variance. These results pave the way for studies at the level of genome features or specific positions, which should elucidate the functional consequences of the patterns observed, and how they translate to the ageing phenotype.

KEYWORDS

**aging, avian senescence, epigenetics, ontogeny, RRBS, *Sterna hirundo***

## 1. Introduction

Senescence is a within-individual decline in survival probability (actuarial senescence) and/or reproductive performance (reproductive senescence) with age. Although the rate and shape of the decline vary both among and within species, this detrimental process occurs in most species across the tree of life (Shefferson et al., 2017). It is hypothesized to have evolved because unavoidable environmentally-driven mortality reduces the strength of selection against poor performance with age (Fisher, 1930; Medawar, 1952; Williams, 1957; Hamilton, 1966). From a genetic point of view, this so-called 'selective shadow' could allow for the accumulation of late-acting deleterious mutations over evolutionary time (mutation accumulation, Medawar, 1952), or for selection favouring alleles with beneficial effects early, but detrimental effects late in life (antagonistic pleiotropy, Williams, 1957). Moreover, early-life investment of limited resources in reproduction over that in perfect somatic maintenance and repair would also be evolutionarily beneficial, such that senescence could

be a 'best-of-a-bad-job' consequence of accumulated, unrepaired damage (disposable soma, Kirkwood, 1977).

Although the classical theories of mutation accumulation and antagonistic pleiotropy assume a purely sequence-level genetic basis to senescence, the disposable soma theory does not. Given its firm foundation in life history theory, resource acquisition and allocation (*sensu* van Noordwijk and de Jong, 1986) are important facets to consider. Both are known to show phenotypic plasticity in response to environmental variation (e.g., Erikstad et al., 1998; Descamps et al., 2016), and experimental manipulation of one of these facets without adjustment of the other is known to affect rates of senescence (e.g., Boonekamp et al., 2014). As such, part of the senescence process is also expected to be underpinned by more flexible, regulatory processes (Wilson et al., 2008). In line with this, numerous studies across taxa have shown that senescence is underpinned by both genetic and epigenetic processes (Sen et al., 2016; Melzer et al., 2020).

Epigenetic processes are those that affect the regulation of gene expression (Holliday, 2006). This regulation is complex and encompasses several mechanisms, a major one of which is DNA methylation, the addition of a methyl-group to the fifth carbon site of a cytosine in a CpG (5'-C-phosphate-G-3') dinucleotide context (Jaenisch and Bird, 2003; Miranda and Jones, 2007; Suzuki and Bird, 2008). Such DNA methylation could lead to transcriptional silencing of genes and repetitive elements, as DNA methylation acts as a ligand for methyl-binding domains, which in turn are targets for further chromatin-modifying complexes such as histone deacetylase complexes or chromatin remodeling complexes (Jaenisch and Bird, 2003; Suzuki and Bird, 2008; Brenet et al., 2011; Miller and Grant, 2013). DNA methylation of gene bodies, however, can lead to increased gene expression (Suzuki and Bird, 2008) or be involved in alternative splicing (Lev Maor et al., 2015). Age-specific DNA methylation has been described for many model species (e.g., Maegawa et al., 2010; Hannum et al., 2013; Tharakan et al., 2020), and the methylation status of specific CpGs has been shown to be a powerful predictor of both chronological and biological age, i.e., to function as an epigenetic clock (Bocklandt et al., 2011). Although age-specific methylation therefore seems the norm, at least in model organisms, the direction of methylation changes with age is harder to predict: both non-directional changes (representing epigenetic drift, Fraga et al., 2005; Tan et al., 2016), and global directional changes (representing global hyper- and hypomethylation, Zampieri et al., 2015; Ciccarone et al., 2018; but see Unnikrishnan et al., 2019) have been reported.

Research on senescence, including its epigenetic underpinning, has so far mostly focused on humans or model organisms kept under controlled laboratory conditions. Extending the taxonomic range and incorporating field studies is crucial to understand the evolutionary ecology of senescence (Monaghan et al., 2008). For this extension, birds are an especially interesting taxon. They have longer life spans relative to their body size than mammals (e.g., Lindstedt and Calder, 1976), and various populations of birds with vastly different life histories have been studied over several decades, such that many basic insights into their senescence patterns can now be obtained (Bouwhuis and Vedder, 2017). Bird genomes are rather compact, show high levels of synteny across species (Zhang et al., 2014), and, similar to those of other vertebrates, are globally methylated (Suzuki and Bird, 2008; Li et al., 2011; Sepers et al., 2019). With respect to age-specific DNA methylation in birds, we are aware of the existence of an epigenetic clock for short-tailed shearwaters (*Ardenna tenuirostris*; De Paoli Iseppi et al., 2019), and of findings of early-life within-individual increases in global methylation in both great tit (*Parus major*; Watson et al., 2019) and zebra finch (*Taeniopygia guttata*; Sheldon et al., 2020) nestlings. However, we are not aware of any study reporting age-specific DNA methylation patterns in avian adulthood, or in avian late-life specifically.

When studying age-specific trait expression, many studies, including most of those producing epigenetic clocks or otherwise studying age-specific differences in DNA methylation patterns, use cross-sectional samples and analysis tools. Patterns revealed by cross-sectional approaches, however, are the result of a combination of within- and among-individual processes. If we aim for understanding the within-individual process of senescence, we need to account for the effect of compositional changes of a population, for example when birds with a certain level of DNA methylation are more likely to die and selectively disappear from the study population (e.g., Vaupel and Yashin, 1985; Forslund and Part, 1995). Mixed-effect models applied to (partly) longitudinal data to specifically test whether within-individual patterns and population-level patterns are the same, or differ, are a powerful analytical tool to do so (van de Pol and Wright, 2009). Across taxa, efforts have increasingly been made to validate and complement findings from cross-sectional analyses of senescence through longitudinal studies (Nussey et al., 2008; Bouwhuis and Vedder, 2017; Gaillard et al., 2017), and this methodological turn is also reflected in studies of human DNA methylation (Bollati et al., 2009; Tan et al., 2016). Longitudinal studies of DNA methylation and ageing in model species and natural populations are, however, rare (but see Lemaître et al., 2021) and needed (Bell et al., 2019).

Here, we report on a longitudinal study of autosomal methylation levels in the common tern (*Sterna hirundo*). Common terns are long-lived migratory seabirds whose patterns of senescence have been the topic of various studies. Although breeders of both sexes show little sign of reproductive senescence - they breed earlier in the year and fledge more offspring as they grow older (Nisbet et al., 2002; Zhang et al., 2015c; Nisbet et al., 2020) - breeding and survival probabilities are known to decline with age (Zhang et al., 2015b; Vedder et al., 2021b). In addition, there is evidence for sex-specific transgenerational senescence, with daughters of older mothers and sons of older fathers suffering from reduced lifetime reproductive success (Bouwhuis et al., 2015). Studies aiming to identify a molecular basis for these within- and transgenerational effects have so far focused on telomere dynamics, and found that: (i) telomeres shorten with age (Bichet et al., 2020); (ii) telomere length is genetically correlated with lifespan (Vedder et al., 2021a); and (iii) paternal age is negatively correlated with offspring telomere length (Bouwhuis et al., 2018). The explanatory power of telomere length, however, is very low for all of these patterns (e.g., 1.1% of phenotypic variation in lifespan is explained by additive genetic variation in telomere length, Vedder et al., 2021a), such that additional mechanisms are expected. To evaluate whether age-specific changes in global DNA methylation could be such a mechanism, we (i) sequenced and *de novo* assembled a high-quality chromosome-scale reference genome and (ii) used it to compare within-individual age-specific changes in DNA methylation at shared sites across the genome. Although rates of within-generational senescence do not differ between the sexes (Zhang et al., 2015a), transgenerational effects are known to be sex-specific (Bouwhuis et al., 2015), such that we also considered sex-specificity of any patterns in autosomal methylation status.

## 2. Materials and methods

### 2.1. Study population

The data we present were collected as part of a long-term individual-based study of a mono-specific common tern colony located on six

artificial concrete islands at the Banter See (53°30'40" N, 08°06'20" E) in Wilhelmshaven, Germany. Fledglings from this colony have been marked with metal rings since 1984, subcutaneously injected with transponders since 1992 (Becker and Wendeln, 1997) and molecularly sexed since 1996 (Becker and Wink, 2003). This marking and sexing, combined with placement of antennae on elevated platforms on the edges of the colony site and around each nest during incubation (shared between both parents), allows for a well-described family structure of all known-sex and known-age philopatric birds (e.g., Moiron et al., 2020).

## 2.2. Reference genome

To create the chromosome-scale reference genome, blood of one adult female common tern was collected in 100% EtOH and stored at −80°C. 30µg of High Molecular Weight DNA (HMW DNA) was isolated from the whole blood sample using an agarose plug protocol of the Bionano Prep Blood and Cell Culture DNA Isolation Kit (cat no. RE-130-10) modified for avian nucleated erythrocytes. Lysates were embedded into agarose plugs, followed by Proteinase K and RNase A treatments and 1X TE drop dialysis purification. Four sequence datasets were generated following the VGP 1.5 pipeline (Rhie et al., 2021): 67.91x Pacific Biosciences (Pacbio) continuous long reads (CLR); 698.35x Bionano Genomics optical maps; 169.30x 10X Genomics linked-reads; and 79.62x Arima Genomics Hi-C Illumina reads.

To create the Pacbio data, DNA was sheared using a 26G blunt end needle (Pacbio protocol PN 101-181-000 Version 05) to approximately ~40kb fragment length. We used 10µg of this fragmented DNA to generate a large-insert Pacbio library using the Pacific Biosciences Express Template Prep Kit v1.0 (#101-357-000). The library was then size selected (>15 kb) using the BluePippin system (Sage Science). The resulting PacBio Library was sequenced on 10 PacBio 1M v3 smrtcells (#101-531-000) on a Sequel instrument with the sequencing kit 3.0 (#101-427-500) and a 10 h movie with 2 h pre-extension time. Unfragmented HMW DNA was used to generate a linked-read library on the 10X Genomics Chromium (Genome Library Kit and Gel Bead Kit v2 PN-120258, Genome Chip Kit v2 PN-120257, i7 Multiplex Kit PN-120262). We sequenced this 10X library on an Illumina Novaseq S4 150 bp PE lane. uHMW DNA was labeled for Bionano Genomics optical mapping using the Bionano Prep Direct Label and Stain (DLS) Protocol (30206E) and 1 flow cell was run on the Saphyr instrument. Hi-C libraries were generated with the Arima Genomics v1.0 2-enzyme protocol (P/N: A510008), according to the manufacturer's protocol and sequenced on Illumina HiSeq X.

The resulting four data types were processed using the VGP v1.5 pipeline (Rhie et al., 2021), which includes: assembling Pacbio contigs using *FALCON v2018.31.08-03.06*; *FALCON-Unzip v6.0.0.47841*; purging false haplotype duplications with *purge_haplotigs v1.0.3+ 1.Nov. 2018*; scaffolding with 10X with *scaff10x v4.1.0;* scaffolding with *Bionano Solve DLS v3.2.1*; scaffolding with Hi-C data with *Salsa HiC v2.2*; filling in gaps and polishing for base call accuracy with CLR and *Arrow smrtanalysis v6.0.0.47841*; and polishing with Illumina short reads with longranger *align v2.2.2*; and *freebayes v1.3.1*. The resulting assembly was then manually curated to fix any errors, using *gEVAL* and Hi-C short read linked-read mapping profiles as described in Howe et al. (2021). *BUSCO v4.1.4* with the bird lineage dataset (*aves_odb10*) was used to assess assembly completeness. The reference genome was submitted to NCBI as GCA_009819605.1, as part of the Vertebrate Genomes Project (VGP).[1] Reference genomes consisting of short-read assemblies (e.g., using Illumina reads) exhibit GC bias, where GC-rich regions such as promoters could be incorrectly assembled or even missing (Kim et al., 2021). Our reference genome, however, has GC-rich promoter regions due to the use of Pacbio long reads (Kim et al., 2021; Rhie et al., 2021).

## 2.3. Blood sampling and methylation sequencing

Blood of breeding common terns was sampled in the years 2013, 2014 and 2017 using larval stages of the bloodsucking bug *Dipetalogaster maximus*. Within 9-14 days of clutch completion of each focal bird in each year, bugs were placed into dummy eggs with holes and placed in the nests (Becker et al., 2006; Arnold et al., 2008; Bichet et al., 2019). After 20–30 min of incubation by the focal bird, "bug eggs" were collected and the focal birds' blood, sucked by the bug, was removed from the bugs' abdomen using a syringe. Upon collection, whole blood was stored in EDTA buffer (2%) in a fridge (3–7°C) for up to 3 weeks, before the red blood cells were transferred to glycerol buffer (40%) and frozen at −80°C. Each bug was used only once to prevent cross-contamination of blood samples.

In total, we obtained 74 samples from 34 individuals making up 17 breeding pairs: 3 breeding pairs (i.e., 6 individuals) were sampled in all 3 years (i.e., with intervals of 1, 3 and 4 years), 14 breeding pairs (i.e., 28 individuals) in two of the 3 years (with a 1-year interval for 4 breeding pairs, a 3-year interval for 9 breeding pairs and a 4-year interval for 1 breeding pair; see Supplementary Table S1 for details). The age of the sampled females ranged between 4 and 20 years, that of males between 3 and 19 years.

Genomic DNA was extracted from each sample and libraries for RRBS were generated as described in Klughammer et al. (2015), following standard steps such as MspI digestion, end-fill-in, A-tailing and size selection. This included the enrichment of the libraries with Pfu-Turbo Cx Hotstart DNA polymerase to allow the assessment of the bisulfite conversion rate. After clean-up and quality control, libraries were sequenced with an Illumina HiSeq 4000 (50 bp SE).

## 2.4. Methylation data selection

Across all 74 samples, individuals were sequenced with an average of 51,482,011 (range: 24,942,071–82,760,425) reads (Supplementary Tables S1, S2). After conversion of unmapped bam files to fastq with *SamToFastq.jar* from *picard v1.118*, the quality of the sequencing reads was checked using *Fastqc v0.11.5* (Andrews, 2010) and *Multiqc v1.8* (Ewels et al., 2016). Spiked-in sequences were used to estimate over- and underconversion using *RefFreeDMA* (Klughammer et al., 2015). *Trimgalore* v0.3.3 (implemented in *RefFreeDMA;* Krueger et al., 2021) was used to remove adaptors to guarantee good mapping and to remove low quality bases (≥20) as well as short read fragments (≥16 bp).

We used *Bismark v0.22.3* (Krueger and Andrews, 2011) to prepare and index the reference genome for subsequent mapping of the bisulfite-converted reads using *Bowtie 2 v2.4.1* (Langmead and Salzberg,

1 https://genomeark.github.io

2012), allowing for one mismatch (score_min L, 0, −0.20). Methylation extraction was conducted with *Bismark Extractor v0.22.3* (Krueger and Andrews, 2011) with the 'ignore' option to remove unmethylated cytosines introduced during the end-repair step. We combined data from the cytosines of both strands at each CpG site with a custom-made python script. The R-package *Methylkit v.1.16.1* in R *v.4.0* (Akalin et al., 2012; R Core Team, 2021) and its function *methRead* were used to load and analyse the methylation calls.

Methylation calls were filtered to those with a minimum coverage of 10 reads per CpG position. CpGs with a coverage >99.9th percentile most likely result from PCR bias and were removed. These two steps were performed using the *filterByCoverage* function in *Methylkit*. Across all 74 samples, there were on average 5,536,718 CpG positions before, and 661,447 CpG positions after, filtering for >10x and <99.9th percentile coverage (Supplementary Tables S1, S2). Methylation call distributions between samples were normalized using the *normalizeCoverage* function implemented in *Methylkit* to reduce the potential bias of systematic oversampling in some samples. We then merged CpG positions of the different samples using the *unite* function to make sure positions were sequenced in at least 70% of the samples (within and across the sexes) to facilitate a longitudinal analysis approach. Following Meng et al. (2010) and Sziráki et al. (2018), sites that showed little or no variation were removed as well, applying a threshold of a standard deviation <0.15. This resulted in 927,490 observations of 15,700 positions, distributed across all autosomal chromosomes (Supplementary Figure S1), which we used to assess autosomal methylation rates by using the number of positions read as our denominator and the number of cases in which the position was methylated as our dependent variable (see *Statistical analyses*).

## 2.5. Statistical analyses

To identify and partition sources of variation in autosomal methylation rates, we used the R package *glmmTMB* (Brooks et al., 2017) to run a generalized linear mixed model (GLMM) with the BFGS algorithm as an optimizer, using the number of methylated and unmethylated Cs (represented by the sequenced Cs and Ts) for each position as our dependent variable, assuming a binomial error distribution (Lea et al., 2017). As fixed effects we added 'sex' (as a two-level class variable using males as the reference category) and age (as a covariate). With respect to the latter, each individual's age was partitioned into an 'average age' and 'delta age' component following van de Pol and Wright (2009). Average age was calculated as the average of all ages at which we assessed a bird's autosomal methylation rate, while delta age was calculated as the difference between the bird's actual age and its average age (i.e., delta age = age − average age). When adding both age variables as covariates, average age represents the among-individual, and delta age the within-individual age effect (van de Pol and Wright, 2009). If the among- and within-individual age effects were to differ, this would indicate that the effect of age among individuals cannot be explained by changes within individuals, thereby revealing age-specific selective (dis)appearance of individuals with certain levels of methylation (van de Pol and Wright, 2009). We additionally included the interaction between sex and delta age in our model to test for sex-differences in the within-individual age trajectory of methylation. Random effects included were 'bird identity' (1–34), genomic 'position identity' (1–15,700 scored as the base-pair-number on our scaffold) and an 'observation'-level random effect (1–927,490 scored at the lowest level of observation, i.e., one value for each genomic position for each individual). The latter was added to (successfully) account for overdispersion detected by the R package *performance* (Lüdecke et al., 2021), whereas the first two were included to account for pseudoreplication caused by repeated sampling of individuals and genomic positions, respectively. Although we could have theoretically added breeding pair identity as a random effect, doing so would only add information when pairs regularly break up and reform among sampling events. Our common tern pairs all stayed together, such that we would not be able to disentangle the effects from pair and bird identify effects.

Because we found a significant interaction between sex and delta age (see *Results*), we also split our data set in one for males and one for females and ran sex-specific analyses including 'average age' and 'delta' age as fixed effects and 'bird identity', genomic 'position identity' and 'observation' as random effects. Moreover, by including positions that were sequenced in at least 70% of the samples, we facilitated a longitudinal analysis approach and a comparison between males and females, but excluded CpGs from the sex chromosome. To also assess age-specificity in methylation rate of the W chromosome, we also ran the sex-specific model for females for the 103 CpG positions we could assess there by including positions that were sequenced in at least 70% of the female samples.

Model evaluation and summary of parameter estimates and statistics were conducted using the R package *parameters* (Lüdecke et al., 2020). Results were plotted using the R-package *sjplot* (Lüdecke, 2018).

# 3. Results

## 3.1. Reference genome

The reference genome was generated using 68x PacBio sequencing reads, Bionano Genomics optical maps, 10X Genomics linked-reads and Arima Hi-C reads. This allowed us to scaffold the assembly to chromosome-level (Rhie et al., 2021) and we successfully assigned 99.3% of the assembled sequence to 25 identified autosomes, two sex chromosomes and the mitochondrial genome, leaving only 95 scaffolds unlocalised. The total length of the primary haplotype assembly was 1.23 Gbp with a contig N50 of 22.0 Mb and a scaffold N50 of 85.5 Mb. The assembly included 96.0% complete single-copy and 0.4% duplicated orthologs according to the BUSCO analysis. Only 1.3% of the gene models were fragmented, and 2.3% missing ($n = 8{,}338$ genes). This represents a high-quality assembly, surpassing the aspired VGP contiguity metrics ~20-fold (Rhie et al., 2021).

## 3.2. Age-specific global methylation

Across the 74 samples, 29,044,662 RRBS reads (~ 55.6% mapping efficiency) could be uniquely mapped to the reference genome (Supplementary Tables S1, S2). The mean bisulfite conversion rate was 99.2% (Supplementary Tables S1, S2). 15,700 autosomal CpG positions occurred in at least 70% of samples within and across the sexes, and across these positions methylation rates ranged from 0 to 1 in both males and females (Supplementary Figure S2). The average predicted methylation levels per sample ranged from 0.437 to 0.752 (mean: 0.562) in females and from 0.424 to 0.777 (mean: 0.627) in males (Figure 1).

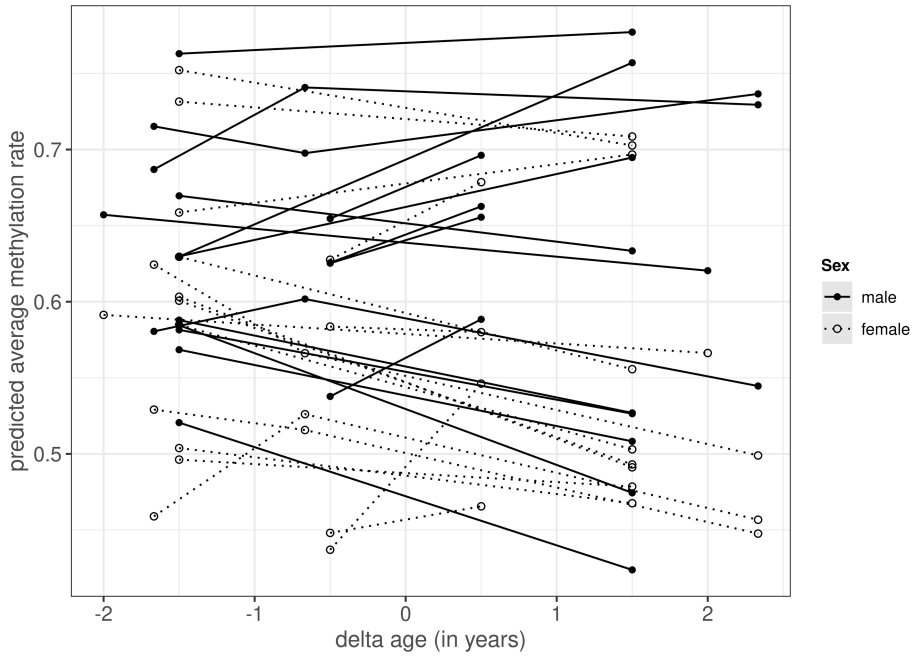


FIGURE 1
Within-individual changes in predicted average autosomal methylation rate for 17 male (black circles with solid lines) and 17 female (white circles with dotted lines) common terns sampled at 1, 3 and/or 4 year intervals. The x-axis, termed delta age, represents the within-individual difference in age (in years) in relation to the average age at sampling (at negative values the individual was younger than its average age at sampling, at positive values it was older). The predicted methylation rate on the y-axis is extracted from the model presented in Table 1, and based on 15,700 autosomal CpG positions.

When analyzing sources of variation in methylation rates across the 15,700 autosomal CpG positions, we found a significant main effect of sex, as well as a significant interaction between sex and delta age (Table 1). The main effect of sex showed males to have higher methylation rates than females, the interaction term indicated that methylation rates showed different age-specificity in males and females. Subsequent sex-specific analyses showed that methylation rates declined with age within individual females, but did not change with age within individual males (Figure 1; Supplementary Tables S3, S4). In the main model (as well as in the sex-specific models, Supplementary Tables S3, S4), the effect of average age was non-significant and the credible intervals of the average and delta age components strongly overlapped (Table 1), suggesting no selective (dis)appearance of individuals based on their autosomal methylation rates.

When assessing the age-specificity of methylation rates in the 103 CpG positions of the female W chromosome, neither average nor delta age was significant (Supplementary Table S5).

When comparing the random effects in the main model, most variance was explained by position identity (Table 1), showing that genomic positions vary in their average rate of methylation. Bird identity, on the other hand, explained variation in methylation rates to a much lesser extent (Table 1), such that there is little evidence for consistent variation in methylation among individuals.

## 4. Discussion

DNA methylation patterns at CpG sites are increasingly used as biomarkers, so-called epigenetic clocks, to predict both chronological and biological age across species and taxa (e.g., Lu et al., 2021). How DNA methylation rate changes within individuals and whether it can explain phenotypic senescence patterns, however, is still largely unknown (Bell et al., 2019). Here, we used blood samples from common terns collected at 1-, 3- and/or 4-year intervals and a longitudinal analysis approach to investigate whether autosomal methylation rates changed with age within individual birds, and whether any change differed between males and females sharing environments and broods. Based on our selection of 15,700 positions, we found female genomes to be generally less methylated than those of males, and to also become even less methylated as these females aged, whereas there was no such age-specific decline of autosomal methylation rate in males. Moreover, we found the estimates for the within- and among-individual components of age to be similar, such that there was no indication for selective (dis)appearance of individuals based on their methylation rate. Finally, we provide evidence for positions in the genome to consistently vary in their methylation rates, whereas evidence for consistent variation among individuals was considerably less.

Our finding of female common terns showing a decrease in methylation rate as they aged fits with findings of global hypomethylation in older compared to younger mammals (Zampieri et al., 2015; Ciccarone et al., 2018; Unnikrishnan et al., 2019). Such methylation loss is thought to partly originate from demethylation of large regions of repetitive sequences, CpG-poor promoters or large hypomethylated blocks of "open sea" regions outside the CpG islands (Bollati et al., 2009; Heyn et al., 2012; Yuan et al., 2015). Whether these changes affect chromatin configuration and thus genome (in)stability, and whether changes in promoter methylation interact with histone modifications and transcription factors to alter expression remains to be investigated (Zampieri et al., 2015; Ciccarone et al., 2018).

Table 1 Results from a generalised linear mixed model with a binomial error distribution testing whether variation in autosomal methylation level is explained by sex (males as a reference category) and the between- (average age) and within-individual (delta age) components of age.

| Parameter | Estimate | 95% CI | *z* | *p* |
|---|---|---|---|---|
| **Fixed effects** | | | | |
| Intercept | 0.6714 | 0.6011, 0.7418 | 18.706 | <0.001 |
| Sex | **−0.0753** | **−0.1248, −0.0258** | **−2.983** | **0.003** |
| Average age | −0.0003 | −0.0056, 0.0050 | −0.113 | 0.910 |
| Delta age | −0.0003 | −0.0031, 0.0024 | −0.234 | 0.815 |
| Sex: delta age | **−0.0041** | **−0.0081, −0.0002** | **−2.083** | **0.037** |
| **Random effects** | | | | |
| Bird identity | 0.0713 | 0.0570, 0.0892 | | |
| Position identity | 1.3413 | 1.3263, 1.3566 | | |
| Observation | 1.2150 | 1.2123, 1.2176 | | |

Estimates and 95% confidence intervals (CI) are provided for each fixed (mean) and random (standard deviation) effect. Significant fixed effects ($p < 0.05$) are presented in bold.

Male terns, in contrast, showed no signs of decreased methylation as they grew older. Although many studies developing epigenetic clocks have assumed age-related changes to be similar across the sexes and used mixed-sex datasets to obtain them [e.g., Horvath et al., 2021; Raj et al., 2021), others have found sex-differences in these clocks (e.g., in some human ethnicities (Horvath et al., 2016), baboons (Anderson et al., 2021) or elephants (Prado et al., 2021)]. Moreover, a rare partly longitudinal study in a wild population of roe deer also showed sex-specific epigenetic clock regions, with an accelerated ageing signal in males, which are known to undergo stronger survival senescence in this species (Lemaître et al., 2021). Combined with our findings, this suggests that tests for sex-specificity of methylation should best be the norm.

Interestingly, male and female terns from our study population do not differ in the onset or rate of senescence in survival or breeding probabilities (Zhang et al., 2015b; Vedder et al., 2021b), nor in their average lifespan (7.4 years for both males and females, Bouwhuis et al., 2015), such that sex-specificity in the ageing process is only found in how parental age affects the quality of the offspring that recruit back into the population [with maternal age negatively affecting the reproductive performance of daughters and paternal age negatively affecting survival of sons (Bouwhuis et al., 2015)]. As such, we did not necessarily expect sex differences in the age-specificity of the birds' autosomal methylation level. The fact that we were able to observe them, raises the question of which site-specific methylation patterns drive the pattern observed on the global level. As mentioned above, global loss is thought to originate from the demethylation of specific regions: repetitive sequences, CpG-poor promoters or large hypomethylated blocks of "open sea" regions outside the CpG islands (Bollati et al., 2009; Heyn et al., 2012; Yuan et al., 2015). CpG island promoters, on the other hand, have been found to show age-specific increases in methylation (Heyn et al., 2012; Day et al., 2013). As such, global demethylation may perhaps be compensated for by such increases in males, but not females. In combination with the fact that we found genomic positions to consistently differ in methylation levels, this stresses the need for moving from the level of global autosomal methylation assessment that we and others (e.g., Watson et al., 2019; Sheldon et al., 2020) have started with, to fully annotating the (common tern) genome and studying patterns across genomic features and focal sites.

Besides finding a sex-specific overall level and within-individual change in autosomal methylation level with age, and significant among-position consistency in methylation level, we found little evidence for consistent among-individual levels of autosomal methylation across years, or for selective (dis)appearance of birds in relation to their methylation level. This suggests that birds differentially change their autosomal methylation from year to year, i.e., show different gaps between epigenetic and chronological age each year, with these changes or gaps perhaps reflecting their individual-specific condition or environment, but not relating to their breeding status (breeding versus non-breeding) in the study population or their local survival. Implementing a random regression analytical framework is data-hungry and not possible with our current dataset, but linking among-year changes in e.g., body mass, other measures of physiology or age-specific reproductive performance seems a promising research avenue, especially when taking the analyses to a site-specific level, such that distinct genotype-methylation-phenotype correlations can be identified.

The strength of our study lies in its longitudinal sampling and analytical approach, which allows us to characterise within-individual changes, rather than infer them from cross-sectional data. At the same time, however, this sampling approach may come with some limitations. Non-destructive, longitudinal sampling in natural populations often relies on using blood as the focal tissue, but how DNA methylation of (in our case) erythrocytes translates to phenotypes mostly remains an open question that needs addressing (Husby, 2020), ideally in experimental study systems. Moreover, because we used RRBS, a high-throughput and low-cost method, to assess methylation, our findings may mostly pertain to methylation of high density CpG regions (Smith et al., 2009; Gu et al., 2011). As such, we may have focally answered the question of what happens at CpG islands during ageing (Beck et al., 2021), something which annotation of the common tern genome will be able to tell. Keeping this in mind, however, our study has provided evidence for a sex-specific overall level and within-individual change in autosomal methylation with age and has shown that CpG positions in the genome vary consistently with respect to their methylation levels, such that future work, changeing the perspective from genome-wide average estimates to the specific genome feature or base pair level, can elucidate whether, where and how much methylation might affect ageing males and females, as such establishing a longitudinal epigenetic clock.

## Data availability statement

The dataset and bioinformatic code presented in this study can be found at Zenodo (10.5281/zenodo.7533890 and 10.5281/zenodo.7493357).

## Ethics statement

The study was performed under licenses of the city of Wilhelmshaven and the Lower Saxony State Office for Consumer Protection and Food Safety, Germany.

## Author contributions

SB collected the blood samples and life history data on the terns. SB and ML designed the study. ML organized sequencing of the samples. BM analyzed the methylation data and performed the statistical analyses with the help of MM. The reference genome was generated as part of the Vertebrate Genomes Project, coordinated by EJ. BH and JM were responsible for sample processing and generated the genome sequence data. CC, GF, and MU-S assembled the genome under coordination of OF. WC, JW, and KH curated the final reference genome. BM and SB wrote the manuscript with input from ML, MM, and EJ. All authors contributed to the manuscript and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.982443/full#supplementary-material

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EDITED BY
Elise Huchard,
UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France

REVIEWED BY
Wenjuan Kang,
Tianjin Medical University Cancer Institute and Hospital, China
Frank Stanczyk,
University of Southern California, United States
Edson Zangiacomi Martinez,
University of São Paulo, Brazil

*CORRESPONDENCE
Frédéric Thomas
frederic.thomas2@ird.fr
Benjamin Roche
benjamin.roche@ird.fr

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# Number of lifetime menses increases breast cancer occurrence in postmenopausal women at high familial risk

Margaux Bieuville[1,2], Dominique Faugère[1], Virginie Galibert[3], Morgane Henard[1], Antoine M. Dujon[1,4], Beata Ujvari[4], Pascal Pujol[1,3], Benjamin Roche[1]* and Frédéric Thomas[1]*

[1]CREEC/CANECEV (CREES), MIVEGEC, Unité Mixte de Recherches, IRD 224–CNRS 5290, Université de Montpellier, Montpellier, France, [2]UMR 7206 Eco-Anthropologie (EA), Muséum National d'Histoire Naturelle, CNRS, Musée de l'Homme, Université Paris Cité, Paris, France, [3]Service Génétique Médicale, Maladies Rares et Médecine Personnalisée, Centre Hospitalier Universitaire de Montpellier, Montpellier, France, [4]Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Waurn Ponds, VIC, Australia

It is increasingly thought that part of human susceptibility to cancer is the result of evolutionary mismatches: our ancestors evolved cancer suppression mechanisms in a world largely different from our modern environments. In that context, it has been shown in cohorts from general Western populations that reproductive traits modulate breast cancer risk. Overall, the more menses women experience, the more at risk they are to develop postmenopausal breast cancer. This points towards an evolutionary mismatch but brings the question whether the reproductive pattern also modulates the breast cancer risk in menopausal women at high familial risk. We thus studied the influence of menses on breast cancer risk in a case–control study of 90 postmenopausal women (including BRCA1/2 and non BRCA1/2) nested within a cohort at high familial risk. We tested the association of the lifetime number of menses and the number of menses before first full-term pregnancy with postmenopausal breast cancer risk using Cox survival models. We showed that the total lifetime number of menses was significantly associated with postmenopausal breast cancer risk and associated with a quicker onset of breast cancer after menopause. Those results align with similar studies lead in general cohorts and suggest that the reproductive pattern modulates the familial risk of developing breast cancer after menopause. Altogether, those results impact how we envision breast cancer prevention and call for more research on how ecological and genetic factors shape breast cancer risk.

KEYWORDS

**evolutionary mismatch, breast cancer, reproductive pattern, survival models, menopause**

## Introduction

Because natural selection usually takes multiple generations to shape fitness-related traits in organisms, individuals living in rapidly changing environments may display, at least temporarily, maladapted features. In humans, such evolutionary mismatches are now known to cause several "modern" diseases (Nesse, 2011) such as obesity or allergies. In addition, most of the major environmental changes are recent in the history of humankind. For instance, investigations on Non-Communicable Diseases (NCDs), as opposed to infectious diseases (Allender et al., 2008) pointed out the role of sedentary lifestyle due to urbanization. In return, sedentarity is known to

be a risk factor for obesity (Lopez and Hynes, 2006). Those evolutionary considerations are important: understanding our vulnerability to NCDs helps to shape public health policies (Nesse and Stearns, 2008).

Although cancer is at least as old as 1.7 millions years (Odes et al., 2016) and has presumably affected humans for a long time (Prates et al., 2011), it is increasingly assumed that part of our susceptibility to it results from evolutionary mismatches between the environment that our cancer suppression systems evolved in and our modern world [see, for instance, Aktipis and Nesse (2013) and Hochberg and Noble (2017)]: in such cases, defense mechanisms are overwhelmed by novel exposures to carcinogens. For instance, regular red meat consumption has been associated with an increased risk of developing prostate (You and Henneberg, 2018) and colorectal (Aykan, 2015) cancers.

In that context, increased breast cancer incidence in controlled-fertility populations could be the result of such evolutionary mismatch. Indeed, a compelling range of reproductive features have been associated with breast cancer risk. For instance, having few offspring and having them late in life is associated with an increased risk of developing breast cancer (MacMahon et al., 1970). You et al. (2018) showed that birth rate variation explains breast cancer incidence variations between countries. In this study, epidemiological indicators such as gross domestic product (GDP), urbanization or overweight, were no longer significantly associated with breast cancer risk once incidence was normalized on birth rate (You et al., 2018).

A lower parity actually results in very high exposure to reproductive hormones: Eaton et al. (1994) calculated that modern American women are, on average, experiencing 450 menstrual cycles in their lifetime (Eaton et al., 1994) whereas Strassmann (1997) showed that Malian Dogon women only experience about 100 menses due to their "natural fertility" (i.e., no contraceptive). Concomitantly, American women are 12 times more at risk to develop breast cancer compared to women from West Africa [who have a similar reproductive pattern according to Strassmann (1999)]. As reviewed by Persson (2000), other traits of the modern reproductive pattern have been associated with breast cancer risk such as ages at menarche and menopause, breastfeeding and exogenous estrogen such as oral contraceptive (hereafter, "OC") and postmenopausal hormone replacement therapy ("HRT"). Hormonal mechanisms partly explain why reproductive traits are tied to breast cancer risk. For instance, the Collaborative Group on Hormonal Factors in Breast Cancer (2012) found that women with mutated estrogen-receptors were more likely to develop tumors during menopause. Additionally, it has been shown that estrogens have a proliferative effect and trigger mammary cell differentiations (Coe and Steadman, 1995). In that same study, Coe and Steadman described the ancestral reproductive pattern as a continuing series of pregnancies and lactation with ancestral women spending most of their lifetime in amenorrhea (implying the absence of ovulation). They hypothesized that departure from this ancestral pattern increased breast cancer risk. Building on observations on the natural fertility population of Dogons in Mali, Strassmann (1999) came to the same conclusion and suggested that allocating more energy to sustain more ovulations could in return decrease the investment in maintenance and protection against proliferative problems. In other words, part of our susceptibility to hormone positive breast cancer might result from an evolutionary mismatch: our exposure to cycling hormones may be higher than our cancer suppression systems have evolved to handle.

Indeed, a second fundamental aspect differs between the ancestral and modern reproductive patterns: exposure to exogenous estrogen and progestogen, OC and HRT. The influence of those exogenous hormones on breast cancer risk is not straightforward. For instance, OCs are supposed to mimic pregnancy and, therefore, could lower the breast cancer risk National Cancer Institute (2018) fact sheet. Conversely, OCs have been associated with an increased risk of developing breast cancer in different studies (see references in the fact sheet). Studies and meta-analyses shedding light on the increased risk due to OC first find a moderate rate increase (Collaborative group on hormonal factors, 2012) and, second, find that the magnitude of the increase depends on both the duration and the type of OC (for instance, progestogen-only OCs are not associated with an increased risk; Mørch et al., 2017). Lastly, levels of exposure must also be compared to those experienced in natural cycles (Lovett et al., 2017), hence differences between progestogens must be asserted. Regarding HRT, the increase in breast cancer risk depends on whether estrogens are administered alone or combined with progestogens and influence marginally decreases after the treatment is stopped (Manson et al., 2013). Overall, OCs seem to moderately increase the risk under certain situations and HRT effects are even more unclear.

To explore the influence of all those reproductive traits, the total number of menses women experience in their lifetime has been used as a metric. Two studies investigated the postmenopausal breast cancer risk associated with the cumulative number of menses and the number of menses before First Full-Term Pregnancy (hereafter "FFTP") in cohorts from the general population (Clavel-Chapelon and E3N Group, 2002; Chavez-MacGregor et al., 2005). These two studies, albeit very similar, slightly differed in their results: Clavel-Chapelon and E3N Group (2002) found a linear risk increase whereas Chavez-MacGregor et al. (2005) suggested that the risk was increasing after a threshold.

Both studies computed the lifetime number of menses by subtracting the time spent in amenorrhea to the reproductive span (between menarche and menopause) and then dividing the result by the average length cycle. It is interesting to note that the lifetime number of menses they compute is a proxy for the "true" number of ovulatory cycles as they consider irregularity to only lead to longer cycles (shorter ones cannot be ovulatory) and as amenorrhea is due to reproductive events known to stop ovulation (such as pregnancy or breastfeeding). However, on the other hand, both studies consider that OCs, while blocking ovulation, create cycles equivalent to ovulatory ones. This is puzzling as (i) more hormones are involved in menstrual cycles than in OCs and (ii) it has been shown that hormonal exposure is different with OCs compared to menstrual cycles (Lovett et al., 2017).

Although paving the way, those two studies did not address the question of how the reproductive pattern influences the risk in women at high risk due to familial history. This is crucial as some genetic loci have been associated with an increased risk to develop cancer but have not been otherwise associated with the phenotypic reproductive traits mentioned above (Warren Andersen et al., 2014). Moreover, known genes are responsible for familial cases of breast cancer (Kuchenbaecker et al., 2017) and can also be associated with reproductive traits. For instance, carriers of mutation in the BReast CAncer (BRCA) gene exhibit differential breast cancer risk according to not only their parity but also the timing of the different pregnancies (Terry et al., 2018).

Here we investigate whether the cumulative lifetime number of menses and the number of menses before FFTP modulates the postmenopausal risk of breast cancer in women at high familial risk. We use this metric as (i) it encompasses the reproductive traits known to modulate breast cancer risk and (ii) it is a proxy for the total number of ovulations that is otherwise inaccessible. We consider that OCs block ovulation and therefore that OCs participate in amenorrhea. Most

importantly, we consider the timespan between menopause and either diagnosis or age at recruitment (in 2017) to address whether women experiencing more menses accelerates the breast cancer onset. We find that the cumulative number of menses is significantly associated with a shorter timespan between menopause and cancer diagnosis whereas the association with FFTP is not significant.

# Materials and methods

## Cohort: Recruitment, data collection, and ethics

Our study sample comes from a cohort of 522 women recruited at the *Centre Hospitalier Université de Montpellier*, France in 2017. The inclusion criteria was family medical history: women were recruited because at least one of their first-degree relatives ($n = 427$) – in majority mothers and sisters – or higher-degree relatives (second degree, $n = 358$ and up to the third degree, $n = 191$) developed breast cancer (216 mothers and 186 sisters) or other reproductive tumors (e.g., ovaries: 55 mothers, 31 sisters). Median age at inclusion was 54 years old. Out of the 522 women, 356 of them had developed breast cancer ($n = 355$ confirmed with medical record). The median age at first breast cancer diagnosis was 40 years old ($n = 337$). Patients lacking age at diagnosis ($n = 19$) were subsequently removed from analysis. Among those 522 patients, 144 carry BRCA1 pathogenic variants and 84 BRCA2 pathogenic variants (44% of our cohort in total).

Patients were asked to answer a survey by phone. Briefly, this survey was made to allow the calculation of the number of menses for each patient so information on the reproductive pattern was collected. Because breast cancer risk may also include a genetic component, patients were asked about their BRCA1 and BRCA2 mutational status, if known. Lastly, family cancer records were assembled according to the relatedness degree and the tumor type.

In this study, we restrict the analysis to a sample of this cohort focusing on postmenopausal breast cancer risk. Therefore, the retrospective case–control analyses were done on postmenopausal women who declared their menopause as "natural" (as hysterectomy without oophorectomy can mimic menopause without blocking the ovarian function and treatments required further assumptions). Patients not reporting ages at menarche and menopause ($n = 7$) or lacking information on their menopausal status ($n = 8$) were removed from the analysis. Eight additional patients were removed when their cancer status (Yes/No) was missing or uncertain. The final sample size is $n = 90$ patients with 49 postmenopausal breast cancer cases and 41 postmenopausal cancer-free controls. Out of the 90 women in the sample, 5 patients were nulliparous and 1 patient had missing FFTP data. Therefore, they were not included in the analysis that focuses on the number of menses before FFTP.

## Reproductive and menstrual variables, mutational status

As explained below, computing the number of menses each patient experienced required to compute the total length they spent in amenorrhea. Each patient's reproductive history was divided into several "blocks" before reviewing the literature on whether those variables are associated with amenorrhea:

(i) "Pregnancy" we considered pregnancy to be 41 weeks long. Puerperium was considered to be between 6 and 8 weeks long as in Wang et al. (2015). We also considered that miscarriages and abortions were followed by a puerperium of the same length ($n = 29$ patients declared at least one abortion and $n = 30$ patients declared at least one miscarriage with two patients reporting a maximum of four events). Patients reported the breastfeeding time for each child. For patients that did not report any length, breastfeeding was considered to induce amenorrhea during a maximum of 6 months per breastfed child and a minimum of 0, assuming the breastfeeding was not exclusive (i.e., use of baby formula) therefore not inducing amenorrhea.

(ii) "Contraception" only hormonal means of contraception were considered. In our cohort, patients used either OCs or implants. If a patient started using hormonal contraception before her FFTP, we considered they used it continuously until this FFTP. This assumption of the model is, on the one hand, supported by the fact that OCs remain the majority contraceptive method in France and are usually used before the first pregnancy and, on the other hand, challenged by the fact that a substantial proportion of women stop taking OCs after 1 year (Vigoureux and Le Guen, 2018)

(iii) "Cycle" including age at menarche, age at menopause, overall regularity and average length of the cycle.

Genetic factors increasing breast cancer risk, such as BRCA pathogenic variants, were considered through a binary variable "mutational status" (Y/N). As shown by You et al. (2018), association of non-reproductive confounders (such as BMI or the living environment) with breast cancer incidence is no longer significant after normalizing by the birth rate. Moreover, BMI were shown to be non-significantly different between cases and controls ($t = 1.2421$, value of $p = 0.22$) and all patients live in the Montpellier area, in an environment that can be assumed to be uniform. Consequently, we chose to only conduct analyses taking into account the reproductive traits, excluding other life variables such as life environment, alcohol consumption or smoking.

## Handling missing answers

In our reduced sample, some patients did not report their age at menarche ($n = 1$), their ages at menopause ($n = 4$), their average cycle length ($n = 8$), how long into the pregnancy before choosing an abortion ($n = 28$) and for how long they took OCs ($n = 8$). Reducing our analysis only to the complete cases would result in too much of a power loss. We therefore chose to fill in missing values using two different methods.

When either the age of menarche or the age of menopause was missing, sample mean values were used as a replacement (respectively, 12.8 and 45.8 yo). Using central measures to replace missing values of a variable relies on two assumptions: the variable sample distribution is symmetric and missing values are randomly scattered in the cohort. To test the first assumption, probability density functions were visually examined before replacement. Additionally, mean values were compared to values in the literature. On the one hand, mean age at menarche (12.8 in our sample vs. 12.6 in the literature) was consistent with literature (Rochebrochard De La, 1999). On the other hand, mean age at menopause was lower than estimates found in the literature (see, e.g., Soules et al., 2001; Weinstein et al., 2003). Lastly, this method was used

in similar studies that also used central metrics as values for single-imputation (Clavel-Chapelon and E3N Group, 2002; Hartz and He, 2013; Khalis et al., 2018).

When a timespan (cycle length, abortion or OC) was missing, a range of plausible values was defined, assuming the distributions were normal. For the cycle average length and the OC, we choose the limit values so that 95% of the sample values belong to the range. For the time to abortion, the lower limit is also the 95% range lower limit but the upper limit is 12 weeks as current French regulation does not allow abortions later than that. This allowed to (i) limit result distortion (Houari et al., 2014) and (ii) to avoid making further biological assumptions.

## General formula for the number of menses

Number of menstrual episodes were computed as follows:

$$N_{\text{menses}} = \frac{\text{reproductive span} - \text{time spent in amenorrhea}}{\text{average cycle length}}$$

For the number of menses before FFTP, the reproductive span is the length between menarche and FFTP and amenorrhea can only be due to OC. For the cumulative number of menses, the reproductive span is the time between menopause and menarche and amenorrhea can be due to pregnancy, breastfeeding or OC.

## Statistical analyses

We first report the average numbers of menses and their standard deviations before testing whether the distributions of the lifetime number of menses and the distributions of the number of menses before FFTP are significantly different between breast cancer patients and controls using the Kolmogorov–Smirnov test and without adjusting for any confounders.

Because it cannot be assumed that controls will never develop breast cancer, survival analyses were conducted to allow for right-censoring. Here we focus on the influence of the cumulative number of menses on the occurrence of postmenopausal breast cancer. Therefore, we use the timespan between menopause and either age at diagnostic (cases) or age at the time of the study (controls) as a time scale to test for the influence of the lifetime number of menses and the number before FFTP. We first compare the Kaplan–Meier curves along the timescale and we then test for potential confounding effects using Cox models. The lifetime number of menses influence is tested along with the mutational status, the parity (Y/N), OC (Y/N), breastfeeding and age at recruitment. All the confounding variables are tested on the same baseline ($p > 0.005$). The number of menses before FFTP influence was tested along with the mutational status and two models were run, adjusting or not for the lifetime number of menses.

Continuous variables have been discretized before being tested in the models: as prescribed by Clark et al. (2003), cutoffs were non-informative (use of quantiles) and more than two categories were created. Two quantiles were used (pooled distribution and distribution in cases) so that comparative results give a first insight on the robustness of the results. All analyses were done in R 3.6.1 (R Core Team, 2020) with the package *Survival* (Therneau, 2020). Testing on the proportionality assumption using the *cox.zph()* function showed no violation of the assumption in either model.

# Results

## Description of the sample

Table 1 below shows how patient and control samples compare.

TABLE 1 Sample characteristics.

| | Cases ($n$ =49) | Controls ($n$ =41) |
|---|---|---|
| Median age at recruitment | 71 yo | 64 yo |
| Median age at menarche | 13 yo | 13 yo |
| Median age at menopause | 50 yo | 50 yo |
| BRCA pathogenic variant | BRCA1 $n$ = 9 | BRCA1 $n$ = 8 |
| | BRCA2 $n$ = 7 | BRCA2 $n$ = 6 |
| No mutation or variant of unknown significance | $n$ = 33 | $n$ = 21 |
| Unknown mutation status | $n$ = 0 | $n$ = 6 |
| Median age at first birth | 24 yo | 24 yo |
| BMI at recruitment (mean ± SD) | 25.19 ± 4.23 | 24.04 ± 4.29 |

## Lifetime menses and menses before first full-term pregnancy: Distribution comparison (midpoint of plausible ranges)

Considering OC use periods as part of time spent in amenorrhea, midpoint means (and standard deviations) for the number of cycles before FFTP were 16.68 (±6.92), 15.59 (±5.32), and 17.94 (±8.29) for pooled distribution, distributions in women with and without cancer, respectively. As seen on Figure 1, the distributions of the number of menses before FFTP are similar between patients with cancer and controls. The Kolmogorov–Smirnov test was not significant (value of $p$ = 0.27). Regarding the lifetime number of menses, looking at the distribution of the midpoint values, means were 389.56 (±113.34), 419.94 (±108.12), 353.25 (±109.87) in the pooled sample, in cancer cases and in controls. The Kolmogorov–Smirnov test was significant (value of $p<0.05$).

## Latency in onset of postmenopausal breast cancer: Cox models (median and limit values in ranges)

To test whether more lifetime menses and more menses before FFTP are associated with an early onset of cancer after menopause compared to controls, we first compared the lifetime number of menses between patients with cancer and controls. As seen on Figure 2, the Kaplan–Meier curves are different: the purple curve is skewed on the right for cancer patients as they experience more menses during their reproductive lifespans (x axis represents the lifetime number of menses). Then, we performed Cox model analyses with the timespan between menopause and either age at diagnosis or age at recruitment (right-censoring) as the timescale. To account for uncertainties in the number of menses, we ran the models with different values (lower and upper limits of plausible range and midpoint values).

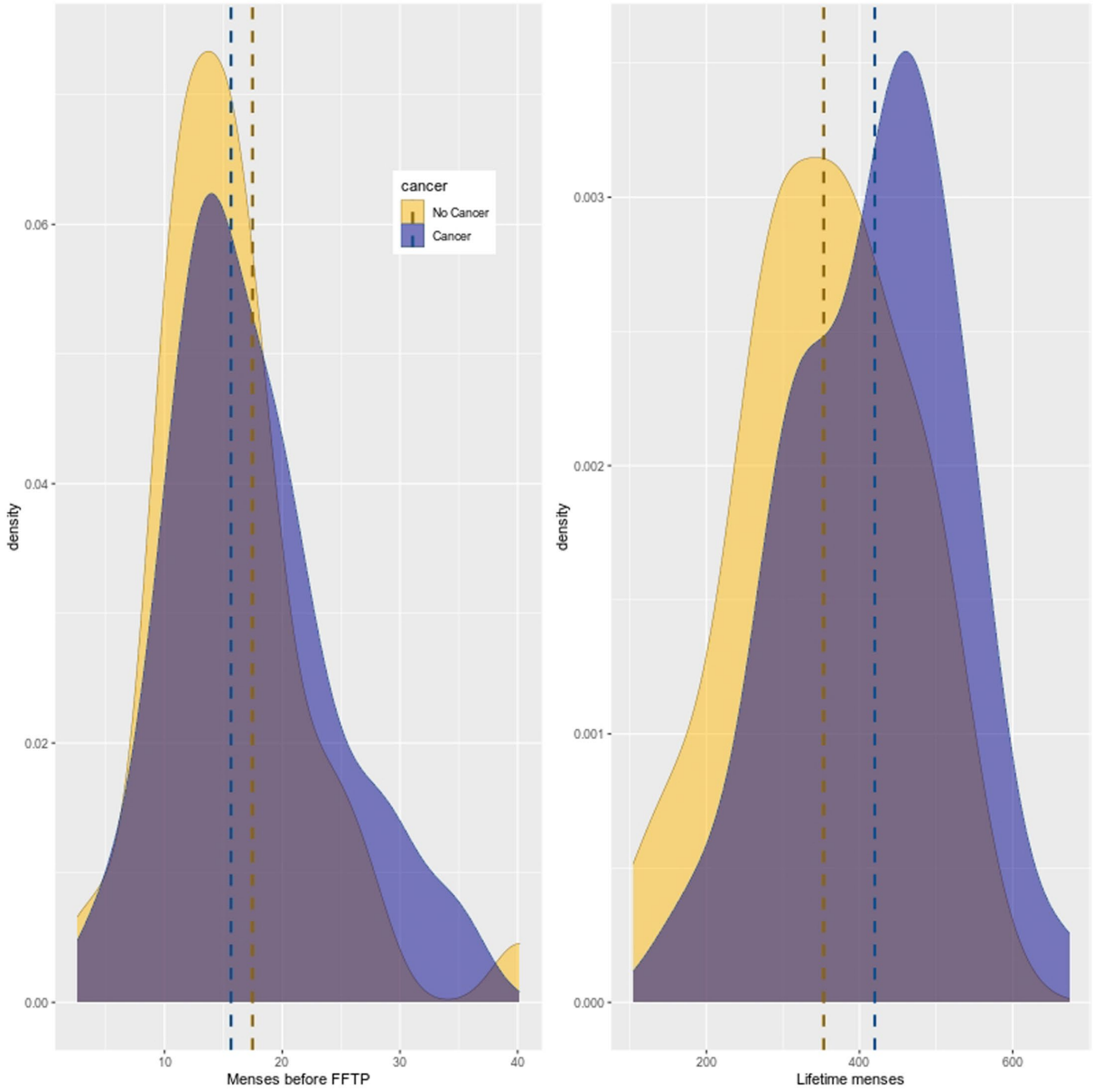


FIGURE 1
Distributions of the number of menses before FFTP and the lifetime number of menses. The distribution of menses before FFTP is similar between cases and controls but distribution of lifetime number of menses is not.

Breast cancer risk according to three levels of the lifetime number of menses ("low" [104;381], "middle" [381;475] and "high" [475;674], computed as the three thirds of the midpoint number distribution in cases. Note that the range notation here shows that patients with the upper limit number of menses for a given range are included in the following range, hence the open bracket) was first tested. Having experienced more menses is positively associated with risk of postmenopausal breast cancer (HR = 1.66 CI95%[0.83;3.33] and HR = 2.24 CI95%[1.13;4.45] for "middle" and "high" levels, respectively) but only the association with the higher category was significant ($p < 0.05$). Additionally, mutational status was added as a confounder and did not yield different results (see Supplementary material S1). When adjusted for OC (Y/N), parity (Y/N), breastfeeding (Y/N) and cycle regularity (Y/N), the model yielded the same results (see Supplementary material S2A).

When the plausible range limit values are used, results are less straightforward. The upper limit values yield that only the "middle" category of lifetime number of menses (Supplementary material S2B) is significantly associated and the lower limit values do not significantly associate any confounders with the latency between menopause and breast cancer diagnosis (Supplementary material S2C). In the same manner, models using the pooled distribution of the number of menses yielded contrasted results (Supplementary material S4) depending on whether the midpoint or the range limit values were assayed. This shows that our results are i) sensitive to the cutoffs and ii) sensitive to the hypotheses made to compute the ranges.

Regarding the number of menses before FFTP, regardless of whether the lifetime of menses was included, both models yielded the same trend: although the category "high" was positively associated, the value of $p$ was not significant. Those results are similar to both Clavel-Chapelon and E3N Group (2002) and Chavez-MacGregor et al. (2005): a positive trend exists but is not significant.

## Discussion

The work presented here investigated the existence of a reproductive mismatch: in postmenopausal women, the risk of breast cancer is increased by greater past exposure to cyclic hormones. We investigated this question using data from a cohort of 90 women drawn from an at-risk population with heavy cancer family history. For each subject, a range of plausible values for the number of menses was computed by considering information on menstrual and reproductive histories.

It has been hypothesized that before the first full-term pregnancy (FFTP), breast cells are highly susceptible to proliferative hormones (i.e., estrogens). Since the question of whether more numerous menses before FFTP is associated with an increase in breast cancer risk is still debated [compare, e.g., Eaton et al. (1994) approach with results reviewed by Persson (2000)], we also investigated how significantly those menses were associated with cancer risk in our cohort. All subjects lived in the area around Montpellier, France, were recruited in the same hospital, surveyed by the same person and had family history of cancer. Therefore, only reproductive confounders have been used in the survival models presented here.

Overall, all the models testing the cumulative number of menses pointed towards the same results: only the lifetime number of menses was positively and significantly associated with a shorter timespan between menopause and cancer diagnosis. This suggests that a greater lifetime exposure to cyclic hormones reduces the latency of breast cancer onset after menopause. On the other hand, the models testing the number of menses before the FFTP yielded a positive but non-significant trend. This results for the number of menses before FFTP should be considered taking into account at least two potential source of bias: i) cycles in the few years after the menarche can be anovulatory (hence, we cannot conclude on the "true" exposure to cyclic hormones) and ii) we assumed that, if patients started taking OCs before the FFTP, they continuously took them. It reduces the number of menses before FFTP and leads to very large ranges of plausible numbers.

This work builds up on the work conducted on large prospective cohorts by Clavel-Chapelon and E3N Group (2002) and Chavez-MacGregor et al. (2005). It has the novelty of investigating how the risk pattern linked to menstrual and reproductive histories will be affected in the context of a population selected based on heavy familial history. It also answers another question: do women who experienced more menses also get breast cancer more quickly after menopause?

Regarding the total number of menses, again, we obtained comparable results to both studies. We found that only the highest range of menses had a significant association. This supports the hypothesis of Chavez-MacGregor et al. (2005) that the risk is likely shaped as a threshold function rather than linear. The similarity in risk pattern between a cohort from the general population and our sample with patients at high familial risk suggests that a high family history burden is not the major risk factor, at least not compared to menstrual and reproductive histories. This is, at first sight, supported by the non-significant positive association of BRCA pathogenic variants with breast cancer risk in our cohort. However, that non-significance can also be the result of a lack of statistical power, so further study on larger sample sizes is required. It also fits well with Genome-Wide Association Studies (GWAS) showing that loci associated with an increased breast cancer risk are not associated with reproductive traits (Warren Andersen et al., 2014). Additionally, the number of menses before FFTP is not significantly associated with the risk despite a positive trend: this advocates even more in favor of this idea that elements of menstrual and reproductive histories influence breast cancer risk in synergy.

Interestingly, the risk pattern was similar in our work compared to the literature despite some differing assumptions. In previous work, whether researchers were counting ovulatory or anovulatory was uncertain. For instance, counting menses during OC treatment suggests a focus on all exposures (not only exposure due to complete ovulatory cycles) but considering puerperium in amenorrhea is considering the cessation of ovarian function. Here, we tried to consider only ovulatory cycles. Because the "true" number of ovulatory cycles was not accessible, we computed a range. It has the advantage of avoiding the need for some biological assumptions, for instance the fraction of ovulatory cycles in the few years before menopause. On the other hand, it also shifted the focus towards endogenous hormones and did not allow us to answer whether OCs protective (and whether, as suggested by Britt and Short (2012), it should be given to nuns as a preventive treatment) or associated to a slight increase in breast cancer risk (Mørch et al., 2017).

First of all, the data required missing value replacement due to the low sample size not allowing for removal. Two strategies have been adopted: mean and interval substitutions. Both strategies assume the same thing: data are missing at random. As explained by Acock (2005), missing data arising from individuals refusing or failing to complete surveys is not entirely random. In our dataset, some of the surveys have been completed by family members of the sampled women. Therefore, we cannot ensure that all missing data are at random. Mean replacement is the simplest way of dealing with missing data but assumes a symmetrical distribution. Additionally, it adds information by increasing the sample size and distorts the distribution by reducing the variance. Assumptions were met for the age at menarche. Regarding age at menopause, it was less straightforward. Interval replacement assumes that data are normally distributed. Once again, this would need to be more thoroughly assessed.

Second, this work presented results obtained on a relatively small sample size. Because Cox models require at least 10 cases per group to yield a decent statistical power (Bradburn et al., 2003), the number of covariates that could be adjusted for in the same model was limited. Lastly, the different models were not as robust as the ones found in literature: $p$-values were sensitive to covariate discretization (using quantiles from either the pooled or the cases distributions).

Third, the work presented here is an application of a prospective model to retrospective data. We computed the number of menses the

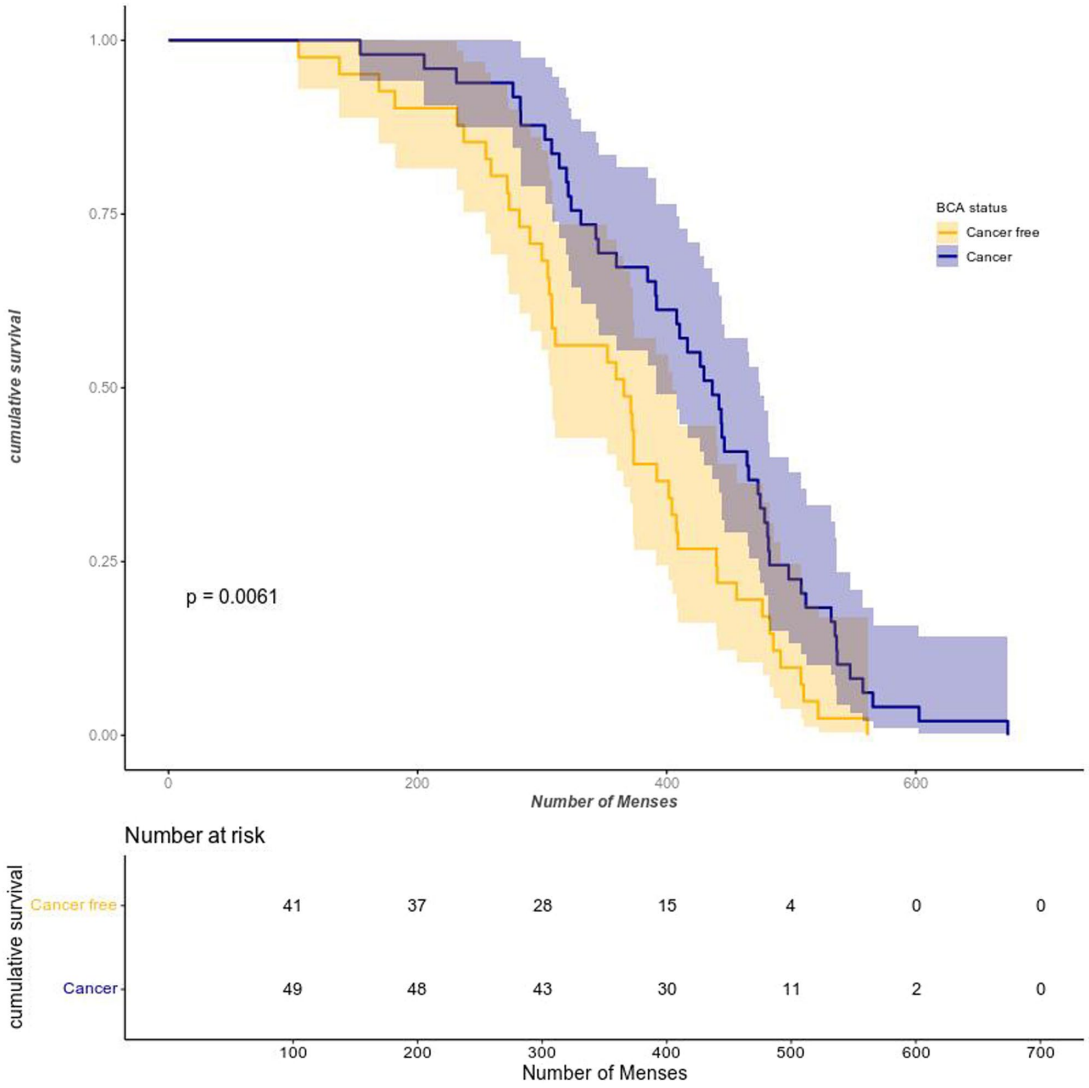


FIGURE 2
Survival as a function of the lifetime number of episodes. The Kaplan–Meier shows that without correcting for any cofactors, cancer patients experienced more lifetime menses than controls. Please note that the horizontal axis represents the lifetime number of menses, not chronological time. BCA status = Breast CAncer.

same way as others did but all subjects who had cancer had it beforehand. Consequently, we cannot ensure the absence of recall bias: medical history has been shown to influence the way individuals remember events. Additionally, women answered about distant past events (Skegg, 1988). The Collaborative Group on Hormonal Factors in Breast Cancer (2012) found evidence that women's answers tend to regress to the mean with further time from the event. Hence, we cannot ensure the absence of recall errors either.

Ultimately, our analysis considered molecular subtypes of cancer altogether whereas reproductive and menstrual traits have been shown to be positively and significantly associated with breast cancer risk for specific molecular subtypes (Aktipis et al., 2014).

This work suggests that the lifetime number of menses is associated with an increased risk of developing breast cancer more quickly after menopause in women at high familial risk. Therefore, high familial risk could be modulated by the reproductive history and the interplay between genetic and reproductive factors should be further studied.

## Data availability statement

The datasets presented in this article are not publicly available as they contain private and medical information. Requests to access the datasets should be directed to margaux.bieuville@cri-paris.org; p-pujol@chu-montpellier.fr; v-galibert@chu-montpellier.fr.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of the Centre

Hospitalier Universitaire de Montpellier. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

MB conducted the statistical analyses. PP, VG, MH, and DF collected the data. BR and FT designed the question and supervised the project. All authors participated in the manuscript writing and approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.912083/full#supplementary-material

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EDITED BY
Jean-Francois Lemaitre,
UMR5558 Biométrie et Biologie Evolutive (LBBE), France

REVIEWED BY
Claudia Fricke,
Martin Luther University of Halle-Wittenberg, Germany
Carl Soulsbury,
University of Lincoln,
United Kingdom

*CORRESPONDENCE
Kristýna Míčková
✉ mickovkr@natur.cuni.cz
Tomáš Albrecht
✉ albrecht@ivb.cz

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# Age-related changes in sperm traits and evidence for aging costs of sperm production in a sexually promiscuous passerine

Kristýna Míčková[1,2]*, Oldřich Tomášek[2,3], Václav Jelínek[2], Michal Šulc[2], Lukáš Pazdera[1,3], Jana Albrechtová[1,2] and Tomáš Albrecht[1,2]*

[1]Department of Zoology, Faculty of Science, Charles University, Prague, Czechia, [2]Institute of Vertebrate Biology, The Czech Academy of Sciences, Brno, Czechia, [3]Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czechia

In many animal species, organismal performance declines with age in a process known as aging or senescence. Senescence typically leads to a deterioration of physiological functionality and can impact the development of primary sexual phenotypes. Sperm production is a complex and costly process that is sensitive to changes in individual physiological state, yet remarkably little is known about age-related changes in sperm performance and aging costs of sperm production. Here we use a non-linear generalized additive mixed models (GAMM) modelling to evaluate age-related changes in postcopulatory sexual traits in the European barn swallow (*Hirundo rustica rustica*), a relatively short lived sexually promiscuous passerine species, where male extra-pair fertilization success has been shown to increase with age. We confirmed a positive relationship between sperm midpiece length and sperm velocity in this species. Within-male changes in sperm morphology and sperm velocity were in general absent, with only sperm length decreasing linearly with increasing age, although this change was negligible compared to the overall variation in sperm size among males. In contrast, the cloacal protuberance (CP) size changed nonlinearly with age, with an initial increase between the first and third year of life followed by a plateau. The results further indicate the existence of a trade-off between investments in sperm production and survival as males with large CP tended to have a reduced lifespan. This seems consistent with the idea of expensive sperm production and survival aging costs associated with investments in post-copulatory traits in this sexually promiscuous species.

KEYWORDS

**senescence, post-copulatory sexual selection, life-history trade-offs, sperm morphology, sperm velocity, cloacal protuberance, *Hirundo rustica***

## Introduction

Aging leads to a deterioration of physiological functionality and reduced individual fitness and reproductive performance (Partridge and Barton, 1993). A central idea of the contemporary theory of aging is the declining force of natural selection with increasing age (Medawar, 1952; see also the antagonistic pleiotropy theory; Williams, 1957). Because individuals typically have limited resources they invest in growth, reproduction and somatic maintenance (Kirkwood and Rose, 1991), this potentially leads to an evolutionary trade-off between reproductive effort early in adulthood and aging, i.e., there is a trade-off between allocation of resources to reproduction versus somatic

maintenance (disposable soma theory of aging, Kirkwood, 1977). So far, evidence for these allocation trade-offs is mostly available for females (Lemaître et al., 2015; Lemaître and Gaillard, 2017). In males, the existing studies evaluating potential trade-offs between reproduction and aging mostly focused on the investment in secondary sexual traits (e.g., Evans, 2003; Robinson et al., 2006), while less attention is currently paid to postcopulatory traits, such as sperm production, performance and morphology reviewed in Lemaître and Gaillard (2017) and Lemaître et al. (2020). This is despite the fact that in sexually promiscuous species, sperm quality and numbers typically determine male fitness reviewed in Snook (2005) and sperm production is presumably an energetically demanding process (Hayward and Gillooly, 2011). Thus, resource allocation to postcopulatory traits may play an important role in early vs. late life investment trade-offs, thereby determining male life-history strategies (Lemaître et al., 2020).

There is considerable variability in the rate of aging among unrelated phylogenetic lineages (Jones et al., 2014), among closely related species (Fletcher and Selman, 2015), or within species among individuals (Lecomte et al., 2010). Aging is typically associated with changes in physiological and behavioral traits, such as foraging efficiency, immune system efficiency, health or individual condition (Reimers et al., 2005; Catry et al., 2006; Angelier et al., 2007). Similarly, individual reproductive performance may deteriorate with increasing age (reproductive senescence; e.g., Reid et al., 2003; Radwan et al., 2005; but see Bouwman et al., 2007). However, given the assumed trade-off between reproduction and somatic maintenance, it has also been hypothesized that individual strategies to optimize lifetime fitness may involve a change of life history allocation based on life expectancy, such that early in life individuals would invest primarily in survival, whereas towards the end of life they would invest more in reproduction at the cost of self-maintenance (Jones et al., 2014). This "terminal investment" hypothesis (Clutton-Brock, 1984) does not predict a deterioration of sperm traits with age but rather an increase of sperm quality and sperm production at terminal age.

Sperm phenotype is an important predictor of male reproductive success in sexually promiscuous species. The length of sperm (and its components) has been shown to play a role in determining sperm fertilization capacity across vertebrate taxa (LaMunyon and Ward, 1998; Knief et al., 2017). Sperm morphology, especially flagellum or midpiece length, can affect sperm velocity, another relevant factor affecting male fertilization success (Firman and Simmons, 2010; Knief et al., 2017; reviewed in Gomendio and Roldan, 2008). Sperm morphometry, and to a lesser extent sperm velocity, is genetically determined (Birkhead et al., 2005; Mossman et al., 2009), but there is evidence of plasticity caused by various factors, such as dietary quality (Støstad et al., 2019), redox state (Tomášek et al., 2017), stage of the breeding season (Edme et al., 2019), social environment (Immler et al., 2010), or immune reaction and health status (Losdat et al., 2011; Svobodová et al., 2018). These observations imply that, similar to precopulatory sexual traits (Andersson 1994), sperm traits may be condition dependent. Moreover, increased sperm production (and large ejaculate volumes) may allow males to transfer more sperm or increase the copulation frequency compared to other males, thus outcompeting males with low sperm production (Schulte-Hostedde and Millar, 2004; Boschetto et al., 2011); however, sperm production seems to be costly, as evidenced by the fact that males adjust ejaculate quality and quantity to the level of sperm competition (e.g., Ramm and Stockley, 2009) and that sperm production is often restricted to short periods of the year when sexually receptive females are present in the population (Calhim and Birkhead, 2007; Lüpold et al., 2012).

If sperm production is costly, it could be assumed that sperm quantity and performance change with age (Lemaître et al., 2020). However, the results of the few studies analyzing the correlation between age and sperm traits are inconsistent. There could be no age-related pattern in sperm sizes (Girndt et al., 2019) or older males produce longer sperm (Gasparini et al., 2010; Langen et al., 2017) or sperm with reduced velocity (Pasqualotto et al., 2005; Gasparini et al., 2010; Meunier et al., 2022). Similarly, old males could produce either higher (Schiavone et al., 2012; Jin et al., 2016) or lower (Pasqualotto et al., 2005; Langen et al., 2017; Meunier et al., 2022) sperm numbers compared to young males. Unfortunately, most available studies are cross-sectional and distinguish only between two age categories (young and old males). Only a few studies, mostly on farm animals, evaluated within-male changes in sperm traits (Dean et al., 2010; Waheed et al., 2015) but focused on a restricted part of individual life cycle (see also Vega-Trejo et al., 2019 for a similar approach in fish). Exceptions are studies on domestic fowl (*Gallus gallus*; Cornwallis et al., 2014) and houbara bustards (*Chlamydotis undulata*; Preston et al., 2011), which showed that the association between age and sperm phenotypes may not always be so straightforward and sperm quality may change nonlinearly with male age. Both studies showed a decline in sperm quality traits with progressing age. In contrast, sperm traits did not decrease with male age in a longitudinal study on ants (Metzler et al., 2018).

In sexually promiscuous songbird species (Passeriformes), old males tend to be more successful than young males in both protecting the within-pair paternity (Bowers et al., 2015; Hsu et al., 2017) and obtaining extra-pair mates (Lifjeld et al., 2011). Although age-related changes in sperm quality and sperm production have been hypothesized to partly account for this phenomenon (e.g., Laskemoen et al., 2010; Lifjeld et al., 2022), data on age-related changes in sperm traits are typically not available for species studied with regard to extrapair fertilization success of young and old males (but see DuVal, 2012; Sardell and DuVal, 2014), and the evidence for age-related changes in sperm morphology and velocity is inconsistent (Laskemoen et al., 2010; Sætre et al., 2018; Edme et al., 2019; Lifjeld et al., 2022). On the other hand, old males may have larger testes than young males, which might indicate intensified sperm production (Laskemoen et al., 2008; Lifjeld et al., 2022). Along with the seasonal increase in testis mass, male passerines produce a pronounced swelling of the cloaca, known as cloacal protuberance (hereafter CP; Wolfson, 1952). The cloacal protuberance is a sperm storage organ the size of which has been shown to positively correlate with testis mass (Birkhead et al., 1993; Laskemoen et al., 2008), the level of sperm competition (Birkhead and Møller, 1998), reflects ejaculate volumes at within-species level (Laskemoen et al., 2010; Girndt et al., 2019) and tends to be positively associated with male fertilization success (Laskemoen et al., 2008, 2010). As with sperm traits, there is contradictory evidence for an association between CP sizes and male age, with some cross-sectional studies reporting no effect (Girndt et al., 2019) or a positive association between CP and male age (Laskemoen et al., 2008).

To date, most available evidence for age-related changes in sperm traits and sperm production available is based on cross-sectional studies (see above) but longitudinal data are urgently needed to assess within-male age-related patterns in postcopulatory traits, as well as to reveal hidden survival and aging costs of potentially expensive and energetically demanding sperm production reviewed in Lemaître et al. (2020). Here we assess age related changes in sperm morphology traits, velocity, and CP sizes in longitudinally observed European barn swallow

(*Hirundo rustica rustica*) males (Michálková et al., 2019; Kauzálová et al., 2022) using generalized additive mixed models (Wood, 2017; Cooper et al., 2021) to construct aging trajectories of each trait across individual lifespans. The studied population of barn swallows is characterized by moderate to high levels of sperm competition, with older males more successful in gaining extra-pair fertilizations than younger males (Michálková et al., 2019). Such pattern could reflect no age-related deterioration of sperm traits in barn swallows, increased investments in sperm production in old males, their increased attractiveness to females (Hsu et al., 2015) but also selective disappearance of low-quality individuals from the population (van de Pol and Verhulst, 2006). In addition, longitudinal data allowed, for the first time in a free-living bird species, to assess the potential survival and aging costs of sperm production.

# Methods

## Study area and general field procedure

Free-living barn swallows were captured with mist nest approximately every 3 weeks from beginning of May to the end of July at four localities in South Bohemia, in the protected landscape area of Třeboňsko - Hamr in Lužnice (49°3′24.217”N, 14°46′9.361″E), Šaloun in Lomnice nad Lužnicí (49°04′07.6”N, 14°42′37.7″E), Břilice (49°01′13.4”N 14°44′17.5″E), and Stará Hlína (49°02′21.2”N 14°49′06.5″E) in the breeding seasons of 2010–2021. All birds were marked with an aluminum National Museum of Prague ring and an individual combination of color rings. The age of birds that were ringed as a nestling at our study area (intensive ringing of the population started in 2008) was known. Birds that were captured un-ringed were assumed as 1-year old that hatched outside our observed populations. This approach has been used in previous studies (Costanzo et al., 2017; Kauzálová et al., 2022) and is possible because of high breeding philopatry and fidelity of barn swallows. Adult birds that did not return to the breeding locality were presumed dead (Costanzo et al., 2017; Kauzálová et al., 2022). Only resident birds (repeatedly occurring on breeding grounds) with a known year of birth and death were included in the analysis.

To obtain CP sizes, we measured the height of male cloaca and its maximum dimension along the two perpendicular axes ($d_1$ – transverse and $d_2$ – longitudinal, see also Laskemoen et al., 2010). The size of CP was calculated by the formula of an ellipsoidal cylinder: height × $\pi \times 0.5d_1 \times 0.5d_2$ (Laskemoen et al., 2008, 2010). CP sized were obtained immediately before sperm collection (see below) to avoid size changes after cloacal massage. Barn swallows usually produce sperm throughout the entire breeding season (April to August). The dataset only includes sexually active males that provided a good quality ejaculate sample and were sampled at the peak of the breeding season (early May to second half of July). Using this dataset, we tested the effect of sampling date (linear and second-order polynomial) on CP size and found no relationship (Supplementary Table S1). All measurements were performed blindly with respect to knowledge of individual age.

## Sperm morphology

Sample of male ejaculate for sperm morphology measurement was taken from each male using a non-invasive method of cloacal massage (Wolfson, 1952; Albrecht et al., 2013). The sample was placed in a 5% formalin solution until the smears were prepared. Using an automatic pipette, 7 μL of each sample were transferred to a slide and allowed to air-dry before rinsing with distilled water to remove impurities. The slides were viewed at 400× magnification using a light microscope (BX51, Olympus, Japan), digital camera (DP71, Olympus, Japan) and imaging software (QuickPHOTO Industrial, Olympus Japan). Passerine sperms have a spiral conformation, the head is helical and the midpiece is distinctly elongated along the flagellum (Humphreys, 1972). Any sperm that did not conform to the characteristic helical conformation was marked as abnormal and was not included in the analysis. However, the number of abnormal sperm was very low (typically <1% sperm cells in the ejaculate). Using QuickPHOTO Industrial software, images of 10 spermatozoa with regular morphology from each ejaculate were taken. Sperm components (head length, midpiece length, and tail length) were measured to obtain an average value of sperm morphology traits for each ejaculate, which was used in further analyses. Total sperm length was calculated as the sum of these three sperm components, while flagellum length was calculated as the sum of the midpiece and tail length. All sperm morphological traits were significantly but rather weakly correlated with each other ($N = 921$ ejaculates, all $r < 0.50$; Supplementary Figure S1; Supplementary Table S2), except for midpiece and tail ($r = -0.73$) and total sperm length and flagellum ($r = 0.97$). Tail is the remaining part of the flagellum where the midpiece does not extend, and, in contrast to the midpiece (Knief et al., 2017), its biological relevance is unclear. Therefore, we did not use this trait in further analyses of age-related changes in sperm morphological traits. We also did not analyze age-related changes in flagellum length, as this was strongly correlated with total sperm length (above).

## Sperm velocity

Samples for sperm velocity analysis were immediately transferred to Dulbecco's Modified Eagle Medium (Invitrogen) prewarmed at 40°C. Then we transferred a small amount of sample onto a prewarmed 4-chambered microscope Leja slide (20 μL deep, Leja, Netherlands). Sperm performance was recorded at 100× magnification using microscope CX41 (Olympus, Japan) fitted with the thermo plate (MATS-U55S Tokai Hit, Olympus), phase contrast, digital camera UI-1540-C (Olympus) and Olympus software QuickPHOTO Industrial. Sperm performance was recorded at multiple locations on the slide to record a sufficient number of sperms.

The recordings were analyzed using the CEROS computer-assisted sperm analysis system (Hamilton Thorne, Inc., United States). In the statistical analysis, the curvilinear velocity (VCL) value was used. This value characterized sperm speed over the entire trajectory by determining speed at each point of the pathway and it is an appropriate measure of swimming speed because of the absence of directional cues (Kleven et al., 2009; Laskemoen et al., 2010). Only progressive tracks were included in analysis (i.e., static and slow tracks were eliminated to remove the potential effect of drift in the chamber; Kleven et al., 2009). Moreover, cases where multiple sperm pathways merged were eliminated and samples containing less than 20 motile sperms were excluded from analyses. Sperm velocity measurements were performed blindly with respect to knowledge of the individual age.

## Statistical analysis

Statistical analysis was performed using R 4.2.0 (R Core Team, 2020). Repeatability was estimated for males with more than one observation in life using the package *rptR* (the number of bootstraps was set to 1.000, Stoffel et al., 2017). Coefficient of variation (CV) was calculated as SD/mean × 100. Associations between sperm morphology and velocity were analyzed using lmer models in the *lme4* (Bates et al., 2014) and *lmerTest* packages (Kuznetsova et al., 2017) with male identity and capture year as a random effect. To explain relationships between selected variables and age we used GAMM in the *mgcv* package with implemented nonparametric smoothing functions because of the possibility that aging trajectories may not follow parametric functions. GAMM are extended versions of generalized linear mixed models which include nonparametric terms and they are also not limited by parametric functions (Wood, 2017). The effect of age was modelled using penalized thin plate regression splines which estimated relationships between age and variables with penalized additive smoothing functions determined by restricted maximum likelihood. The penalized additive value of the number of smoothing functions is the effective number of degrees of freedom (EDF). An effective number of degrees of freedom value 1 indicates that the relationship is linear, a higher value determines the polynomial of the corresponding order (2 quadratic, 3 cubic, etc.). Only one measurement in each year for each male was used in the models. All models also included lifespan as one of the explanatory variables and male identity and capture year as random effects. The use of male identity as random affect allowed us to control for within-individual between-season variation in reproductive traits. Including both age and lifespan in the model allows us to identify within-male changes in sperm traits and sperm production and test selective disappearance of individuals in relation to postcopulatory traits investments. For example, in the presence of selective survival of males with better sperm performance, analyses would give false-positive age-related trends for these traits only because poorer-quality males disappeared (van de Pol and Verhulst, 2006). In case nonlinearity was only weakly supported for a given trait, we also fitted a linear mixed model using lmer in the *lmerTest* package. Since our dataset contained only one male older than 6 years (lifespan 8 years, i.e., one data point for age 7 and 8 years, respectively), the effect of age on sperm traits was also tested after removing this longest living potentially highly influential male and the results are presented simultaneously. To evaluate effect size of age in GAM models, we report the proportion of deviance explained by the variable. The value was calculated as the difference between the proportion of deviance explained by the model that did and did not include age among predictors (Wood, 2017). The significance level was 0.05 for all analyses. Means are reported with their associated standard error (SE).

# Results

## Sperm traits, cloacal protuberance sizes, and their within-male repeatability between seasons

An overview of the phenotypic variation in sperm traits and CP sizes along with sample sizes is provided in Table 1. Repeated measurements (at least two per male in different years) of sperm morphology, sperm velocity and CP sizes were available for 225 males (592 observations), 179 males (453 observations) and 217 males (568 observations), respectively. The within-male between-season repeatability was significant in all sperm traits and ranged between 0.24 and 0.80 (Table 2). While sperm morphology traits exhibited a remarkably high within-male repeatability, sperm velocity was a more variable trait, and the CP size exhibited the highest variability between seasons.

## Sperm morphology and velocity

First, we evaluated the association between sperm morphology traits and velocity (Supplementary Table S3). Velocity was not associated with total sperm length (estimate: $-0.180 \pm 0.189$, $p = 0.342$), head length (estimate: $-1.155 \pm 0.806$, $p = 0.153$) and flagellum length (estimate: $-0.126 \pm 0.195$, $p = 0.520$). Velocity was positively correlated with sperm midpiece length (estimate: $0.514 \pm 0.154$, $p < 0.001$) and negatively with tail length (estimate: $-0.440 \pm 0.134$, $p = 0.001$).

## Age-related changes in sperm traits and cloacal protuberance size

Spline and parametric effects are shown in Table 3. Detailed results for GAMM for each trait (including $R^2$, relevant test statistics and the variance explained by random effects), and proportion of deviance explained by the variable are available in Supplementary Tables S4–S8. There was a tendency for total sperm length to decrease linearly with age (EDF = 1.488, $F = 2.427$, $p = 0.063$). Linear mixed model of the same data also revealed a decrease of sperm length with age with a linear trend best supported (estimate: $-0.127 \pm 0.058$, $p = 0.030$; compared to the second-order non-linear model: $Chi^2 = 1.741$, $\Delta df = 1$, $p = 0.187$; Figure 1A). Results remained unchanged when the exceptionally long-lived male was removed from the analysis (Supplementary Table S9; Supplementary Figure S2). Proportion of deviance explained by age in both models was only 0.2%. Head (EDF = 1.001, $F = 2.061$, $p = 0.152$) and midpiece (EDF = 1.214, $F = 0.060$, $p = 0.910$) showed no age-related trend. Results remained unchanged when the exceptionally long-lived male was removed from the analysis (Supplementary Tables S10, S11).

Sperm velocity showed no change with male age (EDF = 1.003, $F = 0.004$, $p = 0.967$). Results were similar when the exceptionally long-lived male was removed from the analysis (Supplementary Table S12).

The CP size changed nonlinearly with increasing age (EDF = 3.285, $F = 10.416$, $p < 0.001$ for the whole dataset; EDF = 2.851, $F = 13.241$, $p < 0.001$ when the exceptionally long-lived male was removed; Supplementary Table S13). In early life, the CP increases in size and remains stable after the third year of life (Figure 1B; Supplementary Figure S3, respectively). Proportion of deviance explained by age in model was 5.9%, respectively 5.8% when the exceptionally long-lived male was removed. At the same time, CP size tended to be negatively correlated with male lifespan (estimate: $-1.724 \pm 0.969$, $p = 0.076$ for the whole dataset and $-2.138 \pm 1.022$, $p = 0.037$ when the exceptionally long-lived male was removed from the analysis).

# Discussion

In this study we used longitudinal data to analyze within-male age-related changes in selected sperm traits and in sperm production

TABLE 1 Descriptive statistics of total sperm length (TSL), head, midpiece, tail and flagellum length, velocity (VCL) and cloacal protuberance (CP) size.

| Traits | Mean | SD | Minimum | Maximum | CV | *N* |
|---|---|---|---|---|---|---|
| TSL | 87.36 | 2.72 | 71.37 | 95.32 | 3.11 | 921/554 |
| Head | 12.82 | 0.62 | 10.26 | 14.93 | 4.81 | 921/554 |
| Midpiece | 58.58 | 3.27 | 34.98 | 65.24 | 5.58 | 921/554 |
| Tail | 15.96 | 3.79 | 6.18 | 42.85 | 23.78 | 921/554 |
| Flagellum | 74.54 | 2.63 | 60.62 | 81.87 | 3.52 | 921/554 |
| VCL | 95.22 | 13.28 | 51.3 | 150.87 | 13.94 | 776/495 |
| CP | 79.28 | 33.01 | 17.14 | 203.28 | 41.63 | 897/545 |

SD, standard deviation; CV, coefficient of variance; N, number of samples/number of sampled males.

TABLE 2 Within-male between-season repeatability (r) of selected sperm traits (total length – TSL, head, midpiece, tail and flagellum), sperm velocity (VCL) and cloacal protuberance (CP) size for males sampled in multiple seasons.

| Traits | *r* | SE | CI | Value of *p* | *N* |
|---|---|---|---|---|---|
| TSL | 0.783 | 0.0224 | 0.738–0.822 | <0.001 | 592/225 |
| Head | 0.619 | 0.0353 | 0.545–0.681 | <0.001 | 592/225 |
| Midpiece | 0.727 | 0.0281 | 0.670–0.778 | <0.001 | 592/225 |
| Tail | 0.793 | 0.0231 | 0.743–0.829 | <0.001 | 592/225 |
| Flagellum | 0.796 | 0.0222 | 0.750–0.836 | <0.001 | 592/225 |
| VCL | 0.33 | 0.0533 | 0.220–0.426 | <0.001 | 453/179 |
| CP | 0.243 | 0.0491 | 0.146–0.336 | <0.001 | 568/217 |

For details concerning the repeatability analysis see the Methods section. SE, standard error; CI, 95% confidence intervals; N, number of samples/number of sampled males.

TABLE 3 Spline and parametric effects describing age-related changes in sperm morphometry (total length – TSL, head and midpiece), sperm velocity (VCL), and cloacal protuberance (CP) size using generalized additive mixed models.

| Traits | Sample size | Age EDF | Intercept (SE) | Lifespan |
|---|---|---|---|---|
| TSL | 921/554 | 1.488 | 87.640 (0.233) | −0.129 (0.103) |
| Head | 921/554 | 1.001 | 12.829 (0.065) | −0.005 (0.023) |
| Midpiece | 921/554 | 1.214 | 58.951 (0.293) | −0.145 (0.122) |
| VCL | 776/495 | 1.003 | 94.966 (1.623) | −0.157 (0.513) |
| CP | 897/545 | **3.285** | 86.740 (7.339) | −1.724 (0.969) |

Random effects of individual and capture year were included in each model. Full details of each model, including the relevant test statistics, the reference degrees of freedom for each spline term, the deviance and adjusted $R^2$ values, and the variance and number of levels for each random effect, are given in the supplement (Supplementary Tables S4–S8). Sample size, number of observations/number of males; EDF, effective degrees of freedom; Lifespan, number of years an individual lived (provides a test of selective disappearance). Significant variables are highlighted.

(the CP sizes) in a small sexually promiscuous passerine. Previous studies on free-living vertebrates have typically used a cross-sectional approach to study age-related changes in sexual traits potentially involved in post-copulatory mate choice. Moreover, at least in promiscuous passerine species, age was usually assessed at a very coarse scale, distinguishing only two age categories, i.e., young males and old males, making it impossible to analyze changes in sexual traits with age or potential survival and aging costs of sperm production.

Our results indicate that sperm traits exhibit moderate to high within-individual repeatability between seasons. Specifically, morphological sperm traits showed higher repeatability compared to sperm velocity and CP sizes. Lower repeatability of velocity compared to sperm morphology was recorded also in other passerines (e.g., Birkhead et al., 2005; Mossman et al., 2009; Opatová et al., 2016; Sætre et al., 2018). This may indicate that while sperm morphology is under significant genetic control (Birkhead et al., 2005; Knief et al., 2017) and less prone to be affected by environment (e.g., reduced phenotypic plasticity, see also Tomášek et al., 2017), sperm velocity and sperm production may be susceptible to adverse environmental effects, may reflect individual condition (e.g., Simmons and Emlen, 2006) and be influenced by aging processes. Despite differences in within-individual repeatability of sperm morphology traits and sperm velocity, our results suggest a positive association between sperm velocity and midpiece length (and a negative between tail length and sperm velocity). Sperm cells with a longer midpiece-mitochondria (and hence a shorter remaining tail part) are probably able to produce larger amounts of ATP and more energy (Vladić et al., 2002; Rowe et al., 2013) to swim faster (Firman and Simmons, 2010). The positive effect of midpiece length and relative midpiece length on sperm velocity seems to be a general phenomenon in passerine birds (e.g., Laskemoen et al., 2010; Knief et al., 2017; Tomášek et al., 2017; but see Cramer et al., 2015).

No age-related changes in the length of the head and midpiece of sperm were detected in barn swallows, consistent with the relatively high repeatability of morphological traits between seasons (see above). There was evidence for an age-related change in total sperm length, with sperm becoming shorter with progressing age. However, age explained only 0.2% of the deviance in sperm length (Supplementary Table S4), and the within-male sperm shortening (~ 0.13 μm/year) was negligible compared to the inter-male variability in sperm lengths (71 to 95 μm; Table 1). Our results based on a longitudinal approach cannot be directly compared with existing published cross-sectional data. Probably the only available longitudinal study mapping changes in sperm size with age is on ants where age had no effect on sperm size (Metzler et al., 2018). However, cross-sectional studies mostly found no changes in sperm morphology with age of males in

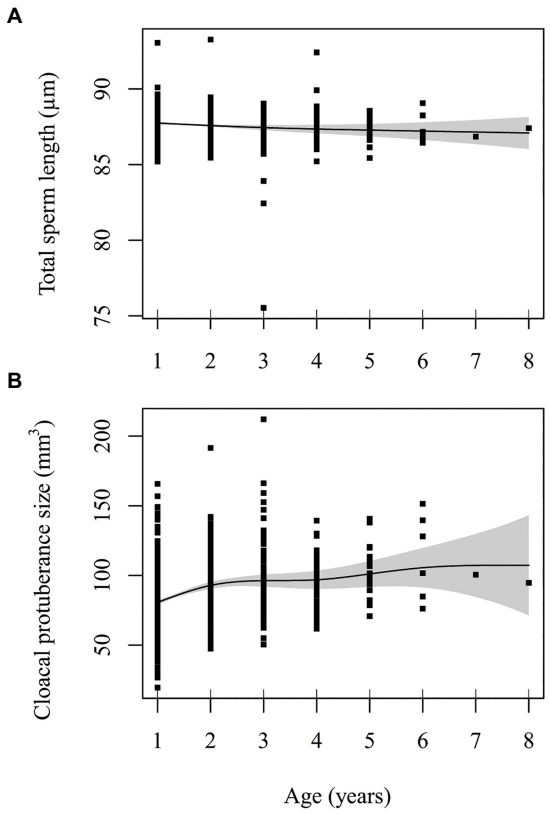


FIGURE 1
Effect of male age (years) on **(A)** total sperm length (µm) and **(B)** cloacal protuberance size ($mm^3$). Line is predicted value from GAMM with 95% confidence intervals. Points are partial residuals from model that included lifespan as a covariate, along with random effects of ring number (ID) and capture year.

various passerine species (Laskemoen et al., 2008; Sætre et al., 2018; Edme et al., 2019; Girndt et al., 2019) including North American subspecies of barn swallows (Lifjeld et al., 2022). On the contrary, some studies, albeit using a limited dataset, found sperm of older males longer than in young males (Cramer et al., 2020). In general, sperm morphology seems to be a relatively stable trait within the life of the male, which is in agreement with the relatively high observed heritability of sperm morphology traits in passerine species (Birkhead et al., 2005; Mossman et al., 2009; Edme et al., 2019). Although some environmental stressors may influence sperm morphology, e.g., oxidative stress (Tomášek et al., 2017), heat stress (Armengol et al., 2015) or pesticides (Uriostegui-Acosta et al., 2014), these stressors may not be important in wild populations or the susceptibility of individuals to these stressors may not be age-related.

Although sperm velocity is a trait exhibiting reduced year to year consistency compared to sperm morphological traits and may be influenced by individual condition or redox state (Tomášek et al., 2017), the lack of association between male age and sperm velocity may reflect an intimate association between sperm velocity and midpiece size, the trait that is also unaffected by age in our population of barn swallows. In cross-sectional studies, sperm velocity did not change with male age in other passerines (Laskemoen et al., 2010; Sætre et al., 2018) but sperm of old males tended to swim faster than sperm of young males in North American subspecies of barn swallows (Lifjeld et al., 2022). However, cross-sectional and longitudinal studies may provide contrasting results as shown in feral fowl (*Gallus g. domesticus*) where no within-male age related change in velocity was found in a longitudinal study, while a cross-sectional approach revealed that older males had reduced sperm velocity compared to young males (Dean et al., 2010).

We found evidence for a previously unknown age-related pattern in CP dynamics in barn swallow males – within individuals, CP sizes increased with age, reaching a plateau when at the age of 3 years, and then remaining stable in size until the male disappeared from the population. The proportion of deviance explained by the age in the model was 5.9% (Supplementary Table S8), suggesting that the effect of age on CP size is not strong, but neither it is negligible. Age related changes in CP sizes in passerines have been documented in several previous cross-sectional studies. In a typical scenario, CP size is larger in older males than young males (Bouwman et al., 2007; Laskemoen et al., 2008; but see Girndt et al., 2019). Age related changes in CP sizes may reflect age related dynamics in testes mass – in North American subspecies of barn swallows testes size increased with age in a cross-sectional study (Lifjeld et al., 2022). CP sizes may reflect ejaculate sizes and investments in sperm production (Laskemoen et al., 2010; Girndt et al., 2019). Thus, an enlargement of CP size with age could explain high extrapair fertilization success of old males in our study population (Michálková et al., 2019).

Lifespan of males was included in all models to evaluate possible selective disappearance (van de Pol and Verhulst, 2006) in relation to postcopulatory traits. Overall, CP size tended to be negatively associated with lifespan and individuals with enlarged CPs were prone to disappear disproportionally from the population, indicating that the production of large ejaculates is costly. It was believed that sperm production is a relatively cheap process (in contrast to egg production; Parker, 1970, 1982). However, comparing energy expenditure on gamete production between the sexes is problematic (Hayward and Gillooly, 2011). Our results indicate an existence of a trade-off between reproduction and survival, and aging costs of sperm production (*sensu* Lemaître et al., 2020). Consistent with costly sperm production is the reduction in testicular size during nutritional stress (Perry and Rowe, 2010), outside the breeding season (Calhim and Birkhead, 2007) or a trade-off between ejaculate quality and either immunity (Simmons, 2012) or pre-copulatory sexual traits (Simmons and Emlen, 2006; Preston et al., 2011). Similar to our study, males in domestic fowl with higher sperm production lived shorter lives (Cornwallis et al., 2014). In contrast, we did not find a relationship between lifespan and sperm morphology traits or sperm velocity. This may indicate producing large sperm quantities, rather than maintaining stable sperm length or sperm velocity, is a costly life-history trait in birds, a taxon where sperm numbers may be a major factor determining male fertilization success (Immler et al., 2011).

To conclude, our results indicate that the production of highly motile sperm with optimal morphology is likely cheaper than the production of large sperm quantities, as only CP sizes were associated with lifespan and underwent a complex dynamic with male age. In general, old males may be more attractive to females as extra-pair partners (but see Girndt et al., 2018), may have easier access to extra-pair copulations than young males (Hsu et al., 2015), and the production of large ejaculates (allowing more copulations) may be rewarding even at the expense of reduced survival. Further studies should aim to reveal the complex relationship between CP sizes, male survival, and extra-pair paternity component of male life-time fitness in sexually promiscuous free-living songbird populations.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

## Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee, The Czech Academy of Science; Animal Care and Use Committee, Charles University in Prague.

## Author contributions

TA conceived the study. KM, OT, and TA designed the study. KM, OT, VJ, MŠ, LP, JA, and TA collected the data. KM and JA analyzed sperm data. KM, OT, LP, and TA performed statistical analyses. KM and TA drafted the first version of the manuscript. All authors contributed to the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1105596/full#supplementary-material

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EDITED BY
Jean-Francois Lemaitre,
UMR5558 Biométrie et Biologie Evolutive (LBBE), France

REVIEWED BY
Matt Dean,
University of Southern California, United States
Geoff A. Parker,
University of Liverpool, United Kingdom

*CORRESPONDENCE
Claudia Fricke
claudia.fricke@zoologie.uni-halle.de

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share senior authorship

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# Timeless or tainted? The effects of male ageing on seminal fluid

Claudia Fricke[1*†], Krish Sanghvi[2†], Mischa Emery[3], Ina Lindenbaum[1], Stuart Wigby[3], Steven A. Ramm[4,5‡] and Irem Sepil[2‡]

[1]Institute for Zoology, Halle University, Halle, Germany, [2]Department of Biology, University of Oxford, Oxford, United Kingdom, [3]Department of Ecology, Evolution and Behaviour, University of Liverpool, Liverpool, United Kingdom, [4]Evolutionary Biology, Bielefeld University, Bielefeld, Germany, [5]UMR 6553, ECOBIO, Université de Rennes, Rennes, France

Reproductive ageing can occur due to the deterioration of both the soma and germline. In males, it has mostly been studied with respect to age-related changes in sperm. However, the somatic component of the ejaculate, seminal fluid, is also essential for maintaining reproductive function. Whilst we know that seminal fluid proteins (SFPs) are required for male reproductive success across diverse taxa, age-related changes in SFP quantity and composition are little understood. Additionally, only few studies have explored the reproductive ageing of the tissues that produce SFPs, and the resulting reproductive outcomes. Here we provide a systematic review of studies addressing how advancing male age affects the production and properties of seminal fluid, in particular SFPs and oxidative stress, highlighting many open questions and generating new hypotheses for further research. We additionally discuss how declines in function of different components of seminal fluid, such as SFPs and antioxidants, could contribute to age-related loss of reproductive ability. Overall, we find evidence that ageing results in increased oxidative stress in seminal fluid and a decrease in the abundance of various SFPs. These results suggest that seminal fluid contributes towards important age-related changes influencing male reproduction. Thus, it is essential to study this mostly ignored component of the ejaculate to understand male reproductive ageing, and its consequences for sexual selection and paternal age effects on offspring.

KEYWORDS

**senescence, reproduction, ejaculates, seminal fluid, germline, oxidative damage, seminal fluid proteins**

## 1. Introduction

Ageing is the time-dependent decline of an organism's biological function (Monaghan et al., 2008), leading to reduced physiological abilities and ultimately death. Ageing results in numerous biological changes that include telomere shortening, accumulation of somatic mutations, loss of proteostasis, mitochondrial dysfunction, and disruption of nutrient sensing pathways (Charlesworth, 1993; Kirkwood, 2005; López-Otín et al., 2013). Organisms also tend to have a lower reproductive output at older compared to younger ages. However, the onset and rate of decline in female fertility varies considerably across taxa, depending on life-history strategies and ecologies of species (e.g., Lemaître et al., 2020b; Campos et al., 2022), making it difficult to generalise patterns of ageing across the tree of life (Jones et al., 2014).

Onset and rate of age-related biological decline and impaired reproductive function varies between males and females (Bronikowski et al., 2022). There has been a long-standing focus on females in life-history research, and studies have only recently begun to consider male reproductive ageing (e.g., Fricke and Koppik, 2019; Comizzoli and Ottinger, 2021; Archer et al., 2022). Evidence suggests that male reproductive ageing can affect male fertilizing ability (Paul and Robaire, 2013; Aich et al., 2021), influence female behavior (Dean et al., 2010; Vuarin et al., 2019), and lead to paternal effects on offspring (Daxinger and Whitelaw, 2012). Male houbara bustards (*Chlamydotis undulata*), for example, produce fewer progeny as they age, and sons of old fathers have greatly reduced sperm numbers (Vuarin et al., 2019, 2021). Other studies show that male ageing can lead to lower sperm quality (Gasparini et al., 2010, 2014; Velando et al., 2011; Cornwallis et al., 2014; Selvaratnam and Robaire, 2016; Monaghan and Metcalfe, 2019; Vega-Trejo et al., 2019; Turnell and Reinhardt, 2020), and quantity (Johnson et al., 2015; Sepil et al., 2020). Additionally, sperm from older males have lower success in sperm competition and fertilize fewer eggs than sperm from younger males, as seen in guppies (*Poecilia reticulata*, Gasparini et al., 2019), zebra fish (*Danio rerio*, Kanuga et al., 2011) and crickets (*Acheta domesticus*, Reinhardt and Siva-Jothy, 2005). Sperm ageing can also affect the quality of offspring (Gasparini et al., 2017), characterized by offspring lifespan (Xie et al., 2018; Wylde et al., 2019), telomere length (Bouwhuis et al., 2018; Noguera et al., 2018; Bauch et al., 2019), development (Preston et al., 2015), reproduction (Bouwhuis et al., 2015; Vuarin et al., 2021), and viability (Tan et al., 2013). While most studies on male ageing have focused on sperm traits, only few have tested for changes in the quality and quantity of seminal fluid with age, and its resultant fitness outcomes. Therefore, whether the reported effects of male ageing are actually driven by changes in seminal fluid rather than just sperm are yet unknown.

Ejaculated sperm are usually surrounded by a cocktail of substances collectively called the seminal fluid (Poiani, 2006; Hopkins et al., 2017). These consist of somatic cells such as immune cells; macromolecules such as carbohydrates, vitamins, minerals; hormones; and seminal fluid proteins (SFPs). Seminal fluid (SF) in most species is made in specialized accessory reproductive cells, tissues, or glands, such as the prostate, seminal vesicle, bulbourethral, and ampullary glands in humans (McGraw et al., 2015). SFPs have been shown to be especially crucial in male and female reproduction; they belong to a range of molecular classes such as antioxidants, lipases, lectins, proteases, and protease inhibitors and have been shown to have a diverse set of functions (Chapman, 2001; Avila et al., 2011; Perry et al., 2013; Ramm, 2020). For instance, SFPs facilitate normal sperm function (Wolfner, 1997), aid sperm storage and male sperm competitiveness (Fiumera et al., 2005, 2007; Goenaga et al., 2015; Patlar et al., 2020), maintain sperm viability (den Boer et al., 2008, 2009; King et al., 2011) and regulate sperm capacitation (Manjunath and Thérien, 2002). But SFPs can also act on attributes beyond sperm, for example, by affecting female reproductive behavior (Chapman et al., 2003; Liu and Kubli, 2003; Bath et al., 2017). Indeed, seminal fluid has been shown to affect female immunity modulation (Short and Lazzaro, 2010), investment in the mating partner's male function in hermaphrodites (Nakadera et al., 2014), female egg-laying behavior (Chapman et al., 2003; Liu and Kubli, 2003), and mating plug formation to prevent female re-mating (Stockley et al., 2020).

The germline is predicted to receive higher protection from somatic ageing (Maklakov and Immler, 2016). Although seminal fluid is produced by somatic tissue it directly interacts with germ cells and thus could play an important role in facilitating interactions between somatic cells and the germline. This could have effects across the Weismann barrier (i.e., despite the germline and somatic tissue being separated early in development, changes in the soma could affect the germline or the next generation; Sciamanna et al., 2019; Bline et al., 2020). Knowing how seminal fluid changes with age and how this can influence sperm, offspring, and female physiological and behavioral responses to mating, in addition to understanding age-related changes in sperm, is essential to gain a complete picture of male reproductive ageing. Here, we first conduct a systematic review on how advancing male age influences the non-sperm components of the ejaculate (i.e., seminal fluid) across animals, and then discuss the impacts this might have on age-specific reproductive success. While the effects of male ageing on sperm have been reviewed elsewhere (e.g., Reinhardt, 2007; Pizzari et al., 2008; Monaghan and Metcalfe, 2019), to our knowledge, this is the first systematic review of how advancing male age affects seminal fluid. As studies differ greatly in their biological and methodological factors, which can modulate or confound male ageing effects, we discuss their possible influence on the conclusions that are reached.

## 2. Systematic review

### 2.1. Literature search and data collection

To understand how male age affects seminal fluid, we conducted a literature search following PRISMA eco-evo guidelines (O'Dea et al., 2021). We used a search string for abstracts, titles, and keywords "*(sfp* OR seminal fluid OR seminal plasma) AND (ageing OR age OR aging OR senescence)*" to identify studies which test how advancing male age affects seminal fluid, using two search engines: SCOPUS and Web of Science (WoS), on December 14th 2021, accessed through the University of Oxford server. The searches returned a total of 738 hits from WoS (year range: 1991 to 2021) and 620 from SCOPUS (year range: 1941 to 2021). After duplicate deletion, which was done using Rayyan (Ouzzani et al., 2016), we obtained a total of 970 unique papers. We then screened the abstracts of these papers using pre-defined inclusion and exclusion criteria (see below), before screening the full-texts to obtain a final list of papers from which relevant data was extracted.

To be retained for full-text screening, the paper had to be a research article (not review or meta-analysis), on any animal, and measure a seminal fluid trait for males of different ages, judged from its abstract. We excluded studies during abstract screening if they were on the wrong topic, did not compare males of different ages, did not have clear ageing data, only covered a small proportion of lifespan (e.g., only included young males), did not measure seminal fluid traits, or only measured seminal fluid during maturation of males (i.e., during juvenile or pubertal stages). The initial screening of abstracts produced a total of 94 studies whose full texts were considered in more detail.

When assessing full texts, to be included in our analysis review, a study needed to: compare males of non-overlapping age groups, compare non-sperm components of the seminal fluid (like oxidative stress enzymes, proteins, hormones, lipids, and macro- or micronutrients), report sample sizes of males in each age group and exact ages (or range of ages) to which males in each age group

belonged. We excluded studies whose full texts were not available (two studies), or which were not in English (three studies). We additionally conducted a scoping search on Google Scholar to obtain additional papers which might have been missed in our systematic screening and search. This was done by using the keywords "seminal fluid protein + aging + ageing + senescence" for each of the following taxa: "bulls," "insects," "pigs," "rodents," "humans," "birds," "mammals," "fish," and searching the first five search result pages for relevant studies.

From all studies which fulfilled our inclusion criteria, we collected information on how male age affected various non-sperm characteristics of the ejaculate, as described in the paper. Additionally, we collected data on factors which could modulate the influence of seminal fluid ageing, such as: male mating history (i.e., whether males were held as virgin or not prior to testing), at which ages males were sampled, what fraction of average lifespan was covered and sampling methodology. The fraction of average lifespan covered is likely to influence whether seminal fluid ageing is detected in a study because ageing trajectories are expected to follow a non-linear pattern, with senescence being more prominent in late-adult life (e.g., Jones et al., 2014; Lemaître et al., 2020b).

Male mating history could influence the ageing of seminal fluid, such that if males are kept virgins, old males would have stored seminal fluid for longer durations, thus have more degraded SFPs and higher accumulation of oxidative damage than mated old males or virgin young males. On the other hand, old virgin males would accumulate higher quantities of SFPs than younger virgin males (Koppik et al., 2018; Sepil et al., 2020). If previously mated males are tested the quantity of seminal fluid produced would depend on the timing of the last mating, number of times the male mated in succession and its rate of replenishment, given that the abundance of SFPs within accessory tissues/glands decreases significantly immediately after a mating event (Hopkins et al., 2019a; Sepil et al., 2019). Furthermore, if mating history is not controlled for, then older males would have mated more times over their life (e.g., Aich et al., 2021), and thus have undergone more rounds of SFP replenishment and thus potentially experienced a higher turnover of the glandular tissue producing the SFP than young males.

Male sampling methodology (if samples are collected longitudinally or cross-sectionally) can also have a large impact on the study outcome. Cross-sectional sampling of males makes age-dependent individual-level deterioration in ejaculate traits with advancing age harder to detect (Nussey et al., 2013), especially if low-quality males selectively disappear (Bouwhuis et al., 2009; Hämäläinen et al., 2014). This non-random age-dependent mortality could lead to biased sampling of males, where younger age classes would have higher variance and might bias estimates of averages in seminal fluid traits compared to old age classes. Thus, cross-sectional studies might underestimate male reproductive senescence, compared to longitudinal sampling measuring the same individuals at different ages.

## 2.2. Summary of studies from the systematic review

Overall, we obtained data from 27 papers through our systematic searches, and seven additional papers from Google Scholar (see Supplementary Table S1 for the full list of included studies). Out of these 34 studies, 14 reported how male age affected SFPs (see Table 1), although some of these studies reported changes in total protein content, while others, changes in specific SFPs only. 10 studies reported data on oxidative damage levels or anti-oxidants present in seminal fluid (henceforth collectively called "oxidative stress," see Table 2). Apart from these two components of the seminal fluid, a smaller fraction of studies assessed the concentration of lipids or lipoproteins (four studies), minerals/vitamins content (four studies), sugar content (two studies), or hormone concentrations (four studies) in the seminal plasma/ejaculate.

The low number of studies dedicated to male age-related changes in the seminal fluid is also reflected in the limited taxonomic breadth, with a strong focus on mammals (see Figure 1). Within mammals, studies were conducted on farm animals, humans, and laboratory rodents (see Supplementary Table S1). For most studies, males were sampled up to around 80% of their average adult lifespan and 50% of their maximum adult lifespan (see Tables 1 and 2 for lifespan sampled by studies; Supplementary Table S2 for sources of lifespan measurements). Another caveat is that non-significant results might go unpublished and it is difficult to estimate this extent, though a number of studies in our set of papers report no changes with age, so we hope the bias is not strong. In the following review, we restricted our discussion to studies that tested for male age-related changes in SFPs and oxidative stress response, as these aspects of the seminal fluid were better represented compared to other ejaculatory components.

### 2.2.1. Age-dependent changes in SFPs

Many studies that measured accessory tissue/gland protein content found an overall decline in SFPs with male ageing (Rezaei et al., 2015; Fraser et al., 2016; Koppik et al., 2018; see also Table 1). However, this pattern becomes less clear when considering studies that quantified individual SFPs or overall compositional changes. Here, some SFPs increased (Santhosh and Krishna, 2013; Simmons et al., 2014; Borziak et al., 2016; Inyawilert et al., 2019; Kant et al., 2019; Westfalewicz et al., 2021), while others decreased in abundance (Marshall et al., 2009; Rezaei et al., 2015; Koppik and Fricke, 2017; Herrera-Cruz et al., 2018; Ruhmann et al., 2018; Sepil et al., 2020; Westfalewicz et al., 2021) with male age. Furthermore, in studies which analyzed the full proteome of the seminal fluid only a small proportion of SFPs changed with age (e.g., Sepil et al., 2020).

Methodologies differed widely between studies, ranging from estimating changes in overall SFP content to reporting individual protein changes. Generally, studies which tended to report increases in SFPs with age (e.g., in *Homo sapiens*, *Bos taurus*, and *Teleogryllus oceanicus*) sampled <50% of the average lifespan of the species (e.g., Simmons et al., 2014; Kant et al., 2019; Westfalewicz et al., 2021). Hence, extending sampling to cover the entire average lifespan is crucial, especially when ageing trajectories are expected to follow a non-linear pattern, with senescence being more prominent in late-adult life.

Most studies on non-human mammals did not report male mating history (virgin or mated) prior to testing. In farm animal studies, older males are likely to have been mated as part of a breeding program, although this was not always explicitly stated. For studies on insects, males were primarily kept as virgins prior to testing. It is known that in *D. melanogaster*, age-related changes in SFPs depend on male mating history (Koppik and Fricke, 2017; Koppik et al., 2018; Sepil et al., 2020). Old unmated males transfer a lower abundance of SFPs in a first mating relative to young males, despite having a higher

TABLE 1 Summary of studies testing the effect of male age on seminal fluid proteins across different taxa as found in the systematic search.

| Study | Species | Proportion LS sampled | Sample sizes | Changes observed in SFPs | Sampling | Mating history |
|---|---|---|---|---|---|---|
| Borziak et al. (2016) | *Gallus gallus* | 1 to 7 years out of 5.5 (avg in wild) and 18 (max) | 16 total | Total of 1,141 SFPs identified, out of which nine changed with age*velocity, and four with age only.<br>Protein tyrosine phosphatase type IVA 1 was present in old males only.<br>Young males had more of SPARC precursor, acetyl-CoA acetyltransferase cytosolic, and ras-related protein Rab-11B compared to old males. | Cross-sectional | Mated but sexually rested |
| Inyawilert et al. (2019) | *Gallus gallus domesticus* | 7 to 24 months out of 60 (avg) and 112 (max) months | 18 total | Proteins with light (72 kDa) molecular weights decreased with increasing age.<br>Mid-weight proteins (90 kDa) increased with increasing age.<br>Heavy proteins (140 kDa) showed no significant change. | Cross-sectional | Unreported |
| Abou-Ahmed et al. (1993) | *Equus caballus* | 7 to 25 years out of 25 (avg) and 47 (max) years | 53 total | Total seminal fluid protein content was highest in middle aged males, and lowest in the youngest and oldest age groups. | Cross-sectional | Mated |
| Westfalewicz et al. (2021) | *Bos taurus* | 2 to 4 years out of 10 (avg) and 25 (max) | 6 total | 17 SFPs differed between young and old males.<br>Older bulls had higher abundances of: glutathione; S-transferase omega 2 (GSTO2); PRDX5; PARK7; superoxide dismutase (SODC), compared to younger males.<br>Younger bulls had higher amounts of: keratin, type II cytoskeletal 59 kDa, component IV (K2C4); outer dense fiber protein 2 (ODF2); tektin-5 (TEKT5) and TBB2B compared to older bulls. | Longitudinal | Unreported |
| Fraser et al. (2016) | *Sus scrofa* | 19 to 42 months out of 66 (avg) and 264 (max) months | 4 total | Overall content of seminal fluid proteins declined with age. Did not identify specific SFPs. | Longitudinal | Unreported |
| Kant et al. (2019) | *Homo spaiens* | 20 to 40 years out of 72 (avg) and 120 (max) years | 6 per age group | 17 protein spots and 10 proteins differed between young and old groups (humans are known to contain ~3,000 SFPs).<br>Glutaredoxin domain containing cysteine-rich protein-2, clusterin, serum albumin, translation initiation factor IF-2 like, ecto-ADP-ribosyltransferase 4, CB1 cannabinoid receptor-interacting protein 1, serotransferrin were found in higher abundance in older males compared to younger males.<br>Alternative protein RRT-34 and protein Unc-119 homolog A were found in lower abundance in older age samples compared younger males. | Cross-sectional | Mated |
| Simmons et al. (2014) | *Teleogryllus oceanicus* | 4 to 20 days out of 74 (avg) and 135 (max) days | 57 total | Total of 27 distinct SFPs identified.<br>Total protein content did not vary with age.<br>ToSfp014, ToSfp025, ToSfp007 (Trypsin-like serine protease), ToSfp017, ToSfp011, ToSfp026, ToSfp005 (Dipeptidase), ToSfp027 (apyrase), ToSfp001, ToSfp024 (carbonic anhydrase) increased with age.<br>Other SFPs did not change significantly with age. | Cross-sectional | Virgins |

*(Continued)*

TABLE 1 (Continued)

| Study | Species | Proportion LS sampled | Sample sizes | Changes observed in SFPs | Sampling | Mating history |
|---|---|---|---|---|---|---|
| Koppik and Fricke (2017) | *Drosophila melanogaster* | 7 to 42 days out of 45 (avg) and 110 (max) days | 10 per age group | All five SFP genes tested decreased in expression with age: Acp26Aa, Acp29AB, Acp36DE, SP and Acp62F. | Cross-sectional | Mated and unmated treatments |
| Sepil et al. (2020) | *D. melanogaster* | 7 to 35 days out of 45 (avg) and 110 (max) days | 80 per age group | 117 SFPs identified, out of which 40 changed with age. Focused on six functionally important SFPs. Acp62F, Semp1, and Acp26Aa decreased with age. Acp70A [sex peptide], Acp36DE, and CG9997 showed no change with age. Age-related accumulation of SFPs in unmated males, but reduced transfer. No change in SFP abundance or transfer with age in frequently-mating males. Evidence of age related post-translational modifications in some SFPs. | Cross-sectional | Mated and unmated treatments |
| Rezaei et al. (2015) | *D. melanogaster* | 2 to 53 days out of 45 (avg) and 110 (max) days | 20 per age group | Overall seminal fluid amount decreased with age. Did not measure specific SFPs. | Cross-sectional | Virgins |
| Ruhmann et al. (2018) | *D. melanogaster* | 4 to 42 days out of 45 (avg) and 110 (max) days | 18 per age group | Measured two SFPs: sex peptide and ovulin. Sex peptide decreased in old males, ovulin levels did not change with age. | Cross-sectional | Mated |
| Herrera-Cruz et al. (2018) | *Anastrepha ludens* | 8 d to 72 days out of 50 days (avg), 1 year (max) | 20 per age group | Old males had lower overall protein content in their testis (but not accessory glands) compared to young males. | Cross-sectional | Virgins |
| Marshall et al. (2009) | *Allonemobius socius* | 5 to 40 days out of 35 days (avg) and 100 days (max) | 42 total | Protein X (trypsin like serine protein) reduced with male age. | Cross-sectional | Virgins |
| Santhosh and Krishna (2013) | *Drosophila bipectinata* | 2 to 47 days out of 58 days (avg) and 200 days (max) | 50 per age group | Overall SFP quantity increased with male age. | Cross-sectional | Virgins |

Proportion lifespan (LS) sampled is given in relation to reported average lifespan (avg) or maximum (max) recorded lifespan (sources for those numbers can be found in Supplementary Table S2) for each species.

abundance of SFPs in storage, whereas old frequently mated males show no change in either transfer or storage (Sepil et al., 2020). Thus, mating history has the potential to influence the results reported in studies which do not control for it. We suggest future studies should adopt a fully factorial design to test for effects of mating history on seminal fluid ageing and use young and old males both as virgin and mated males, and ideally control for mating number.

In most studies, samples were acquired from the male directly (e.g., *via* dissection or masturbation), but whether this correctly represents what would be transferred to females in a natural ejaculate is uncertain, especially when males have the potential for strategic ejaculation (Wedell et al., 2002). Moreover, the vast majority of studies were cross-sectional. It would be ideal to conduct longitudinal studies in species where males do not need to be sacrificed to extract their ejaculate and a large cohort of males can be followed across their lifetime.

### 2.2.2. Age-dependent changes in oxidative stress responses

Overall, the enzymes involved in protecting against oxidative damage decreased significantly in the seminal fluid with advancing male age (see Table 2). The three studies that measured both enzyme abundance and oxidative stress in the seminal fluid found oxidative stress markers increased in older males. Specifically, all studies which measured antioxidant content in the seminal fluid (e.g., TSOD, MnSOD, CuZnSOD, TGSH, CAT) consistently reported a decline in older males compared to younger or middle-aged males. Additionally, an oxidative stress marker was found in higher quantities in older male seminal fluid compared to younger males in two studies (El-Gindy and Zeweil, 2017; Kara et al., 2019). Notably, all these oxidative stress studies used mammals, so we cannot judge whether this is a pattern also seen in other animal groups. None of these studies reported the mating history of the males, and only one study sampled males longitudinally (Fraser et al., 2016).

## 3. Discussion

Here, we systematically reviewed how the non-sperm components of the ejaculate (i.e., seminal fluid) changed with male age. Sperm ageing has been a major focus of previous studies, while seminal fluid has not been studied as extensively. This is highlighted

TABLE 2 Summary of studies found in the systematic literature search that focus on male-age dependent changes in antioxidants, oxidative stress biomarkers and reactive oxygen species in male ejaculates/seminal plasma.

| Study | Species | Proportion LS sampled | Sample sizes | Changes observed in oxidative stress | Sampling | Mating history |
|---|---|---|---|---|---|---|
| Vince et al. (2018) | *Bos taurus* | 2 to 10 years out of 10 (avg) and 25 (max) | 9 young, 9 old | Antioxidants such as TSOD, MnSOD, CuZnSOD, TGSH, CAT all higher in young males. Oxidative stress was higher in old males. | Cross-sectional | Unreported |
| Ahmad et al. (2020) | *Bos taurus* | 3 to 10 years out of 10 (avg) and 25 (max) | 6 young, 6 old | Younger bulls had higher total antioxidants. For catalase and malondialdehyde, there was no sig. difference. | Cross-sectional | Unreported |
| Majić Balić et al. (2012) | *Bos taurus* | 2 to 10 years out of 10 (avg) and 25 (max) | 9 young, 10 old | Season dependent changes in antioxidants: For total glutathione peroxidase (T-GSH-Px), young bulls had more in all seasons. For glutathione peroxidase (Se-GSH-Px), protein carbonyl content (PCC), young males had more in 3/4 seasons. | Cross-sectional | Unreported |
| Kelso et al. (1997) | *Bos taurus* | 2 to 9 years out of 10 (avg) and 25 (max) | 4 in each of the three classes | For both antioxidants measured, glutathione peroxidase and superoxide dismutase, younger males had more than older males. | Cross-sectional | Unreported |
| Noguera et al. (2012) | *Gallus gallus* | 1 to 4 years out of 5.5 (avg in wild) and 18 (max) | 6 young, 15 old | Decrease in antioxidants such as -SH group of proteins, uric acid, vitA, vit C, vit E in old males. | Cross-sectional | Unreported |
| El-Gindy and Zeweil (2017) | *Oryctolagus cuniculus* | 9 to 42 months out of 24 months (avg) and 150 months (max) | 18 young, 18 old | Aspartate transaminase showed no significant change with age. Antioxidants decreased in old males. Oxidative stress marker malondialdehyde increased sig in old males. | Cross-sectional | Unreported |
| Kara et al. (2019) | *Mus musculus* | 3 to 24 months out of 24 (avg) and 48 (max) months | 14 young, 21 old | Antioxidants glutathione peroxidase and reductive glutathione decreased in older males. Oxidative stress marker malondialdehyde increased in old males. | Cross-sectional | Unreported |
| Fraser et al. (2016) | *Sus scrofa* | 19 to 42 months out of 66 (avg) and 264 (max) months | 4 in total | Antiperoxidant activity lower in older animals. Antioxidant L-glutathione concentration peaked at mid age 19–30 mo, and declined in older animals. | Longitudinal | Unreported |
| Waheed et al. (2013) | *Equus caballus* | 4 to 22 years out of 25 (avg) and 47 (max) years | 6 in each age group | Antioxidant glutathione peroxidase highest in middle aged males, and lower in oldest and youngest males. | Cross-sectional | Unreported |
| Takemura et al. (2014) | *Rattus norvegicus* | 15 to 75 weeks out of 124 (avg) and 187 (max) weeks | 4 to 5 in each group | DJ-1 antioxidant decreased with age. Cu/ZnSOD antioxidant decreased with age. | Cross-sectional | Unreported |

Proportion lifespan (LS) sampled is given in relation to reported average lifespan (avg) or maximum (max) recorded lifespan (sources for those numbers can be found in Supplementary Table S2) for each species.

by the limited number of studies and taxa found in our systematic review, with the majority of studies either probing at age-related changes in SFPs or oxidative stress. Below, we discuss how the age-dependent changes in seminal fluid components found in our systematic review might influence male reproductive ageing, suggest some hypotheses, and discuss why the omission of seminal fluid and its associated somatic tissue is an important oversight in evolutionary and ecological research.

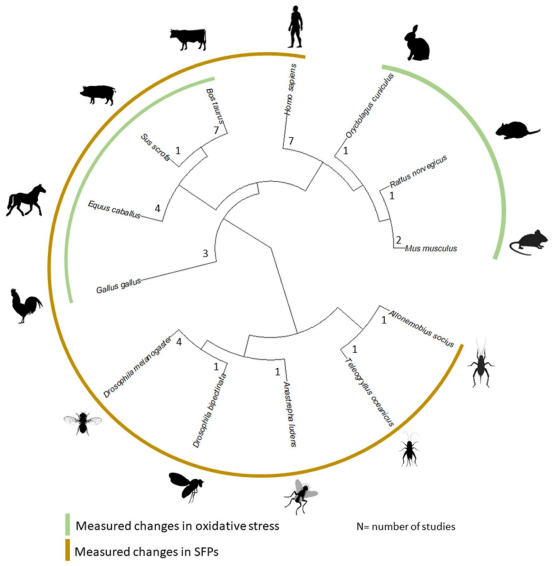


FIGURE 1
Phylogenetic distribution of all species in our review across 34 studies along with the number of studies on each. Species which had data reported for age-dependent changes in SFPs and oxidative stress are marked. Images to generate the figure were taken from the Open Tree of Life.

## 3.1. Seminal fluid protein ageing

We found some heterogeneity between studies in age-related SFP changes. This could be due to studies reporting quantitative changes in a set of proteins only (rather than all the proteins), or due to specific proteins responding differently to age based on their function or tissue-of-origin (Borziak et al., 2016; Sepil et al., 2020). Proteomics techniques that quantify the whole ejaculate are needed to better elucidate these biological patterns and with the advance of molecular approaches and particularly proteomics, this will become ever more feasible for a range of taxa.

Confounding factors could also explain some of the inconsistencies observed between studies. For instance, male mating history could have a large influence on SFP quantity changes as explained in the methods section above. Another caveat in comparing studies is that males are not always sampled up to old age and so an important fraction of the ageing trajectory is missed. This could be a serious bias, potentially compounded by there being stronger selection to maintain functionality earlier in life, meaning realised phenotypes may in part represent compensatory adaptive responses to the onset of seminal fluid ageing. Disentangling age-related changes from compensatory responses would require sampling beyond the 50% average lifespan into older ages, when the latter responses are expected to wane as the strength of selection declines.

While studies in our review rarely directly discuss the functional importance of the changes in observed SFPs, below, we suggest testable hypotheses for how male seminal fluid ageing might have functional consequences. Overall, studies in our review show changes in specific SFP abundances which are known to influence male fertilization success as well as a variety of female responses. For instance, older male *D. melanogaster* are less able to delay female remating and stimulate egg laying compared to younger males (Koppik and Fricke, 2017; Ruhmann et al., 2018; Sepil et al., 2020). Similarly in *Aedes aegypti* mosquitoes, older males are less able to prevent female remating (Agudelo et al., 2021). These responses are largely mediated by SFPs in *D. melanogaster* (Chapman et al., 2003) and the expression of functionally important SFPs declines with age (Koppik and Fricke, 2017). Therefore, it is possible that the decline in SFPs are driving the changes in female post-mating behavior. For instance, Sepil et al. (2020) found a significant age-related increase in SFP abundances in the accessory glands of unmated males, but no change in SFP abundances in the accessory glands of frequently-mated males. Yet, the authors also found that female egg laying behavior and remating affinity changed as a function of male age following matings with spermless males, hence the seminal fluid alone does contribute to the decline in reproductive function with male age. SFP transfer data can partially explain these findings. While there is no age-related decline in SFP abundances in the accessory glands, old unmated males transferred a lower quantity of SFPs to females compared to younger unmated males. There was no age-related change in the quantity of SFPs transferred to females for frequently-mated males, so it is likely that changes in SFP quality rather than quantity explain the decline in reproductive function with age in this group of males.

Apart from affecting male ability to induce female post-mating responses, age-related changes in seminal fluid might also affect sperm traits. For example, in the jungle fowl *Gallus gallus*, age-related changes in proteins which affect sperm velocity were detected (Borziak et al., 2016). Thus, the decreased ability of older males to gain paternity under sperm competition and fertilize eggs may be driven by changes in SFPs rather than changes in sperm *per se*.

While it can be difficult to pinpoint the precise changes responsible, it is becoming increasingly possible to manipulate the expression of individual SFPs to better understand how particular SFPs affect female post-mating behavior and sperm competition. For instance, using a combination of proteomics and RNAi, Marshall et al. (2009) identified a single accessory gland-derived ejaculate protein in the ground cricket *Allonemobius socius* that influences female egg-laying and declines in expression with male age. Hence, this protein is a prime candidate to explain the waning ability of males to induce female egg laying as the male ages. However, the link between seminal fluid expression and female responses is not necessarily straightforward. RNAi knockdown studies in other taxa have demonstrated that suppressing the expression of individual SFPs can have both positive and negative impacts on fitness-relevant traits such as female fecundity (Xu et al., 2013; Weber et al., 2019), though this may in part reflect the difficulty of measuring fitness components under realistic conditions. A further limitation of many such knockdown or knockout studies is that they tend (often of necessity) to consider only one or a few seminal fluid proteins, whereas in reality the seminal fluid proteome is a highly integrated unit whose individual components co-vary in their expression (Mohorianu et al., 2018; Patlar et al., 2019).

## 3.2. Effects of male age on seminal fluid quantity versus quality

In addition to changes in the abundance of individual proteins or changes to the composition of the seminal proteome, ageing can

potentially impact seminal fluid through alterations to protein quality. A loss of protein homeostasis – proteostasis – is a well-known feature of ageing, characterized by a failure of chaperones, stress-response factors, and protein degradation machinery to respond to stress and prevent protein misfolding (Labbadia and Morimoto, 2014). The role of failing proteostasis in loss of SFP quality in ageing males is currently unclear but has the potential to impact ejaculate function.

Work in *D. melanogaster* suggests that factors other than SFP *quantity* may be responsible for the decline in seminal fluid-mediated functions with male age (Sepil et al., 2020). Aged males, that are known to have compromised fertility and reduced seminal fluid function, still appear capable of levels of seminal proteome production and transfer that are similar to young males (Sepil et al., 2020). However, several proteins in aged males show evidence of qualitative changes *via* mass shifts on Western blots (Sepil et al., 2020). While it remains to be investigated how widespread age-related changes in SFP quality are and what the functional consequences are, it nonetheless raises the possibility that a decline in SFP functionality with age is primarily related to proteostasis loss, rather than diminishing amounts of SFPs.

## 3.3. Ageing of seminal fluid producing reproductive tissues

While our systematic review showed general age-related declines in SFPs, how the somatic tissues which produce SFPs are affected by ageing across taxa still remains unclear. Generally, the size of prostates/accessory glands tends to increase as males grow older (Jin et al., 1996; Atalan et al., 1999; Rezaei et al., 2015; Reyes-Hernández and Pérez-Staples, 2017), but shrinkage with age was also reported in few studies (Mazeed and Mohanny, 2010; Santhosh and Krishna, 2013). However, the overall size of the organ does not necessarily predict protein content, as found in *A. ludens* (Herrera-Cruz et al., 2018) and *D. bipectinata* (Santhosh and Krishna, 2013). In humans, the increase in prostate size is known as benign prostatic hyperplasia (Berges and Oelke, 2011; Zhang et al., 2013), however prostate size varies among ethnic groups and so does the rate of change with age (i.e., Bolivian Tsimane, Trumble et al., 2015) or the occurrence of enlarged prostates in older males (Mubenga et al., 2020). Some theory predicts that the enlargement of the prostate is a side-effect of cellular hyperfunction that causes ageing of this tissue (Blagosklonny, 2021). The hyperfunction theory of ageing proposes that suboptimal nutrient-sensing molecular signaling in late-life causes ageing *via* excessive biosynthesis, as opposed to energy-tradeoffs (Lind et al., 2019).

## 3.4. Impact of male age on oxidative stress

The studies we reviewed consistently found that antioxidant quantity in the seminal fluid decreases with increasing male age, while oxidative stress markers tend to increase in the seminal fluid as males age. Reactive oxygen species (ROS) are unstable, free radical compounds and are required for vital cellular processes (Finkel and Holbrook, 2000; Hajam et al., 2022), but can also be deleterious to cells. For instance ROS play a role in sperm activation and changing sperm motility, e.g., in humans (Aitken et al., 2022) with the potential to influence male reproduction (Mannucci et al., 2022). However, work in *D. melanogaster* shows that while older males have higher metabolic rates in their sperm, ROS production is actually lower in these sperm (Turnell and Reinhardt, 2020).

Antioxidants, on the other hand, play a key role in stabilizing free radicals generated as part of cellular processes (Hood et al., 2019), and an imbalance between antioxidants and ROS causes oxidative stress. Oxidative stress has been shown to influence sperm homeostasis and can cause sperm DNA damage thus affecting male fertility (Mannucci et al., 2022) and has been shown to be key in regulating various intracellular pathways related to sperm, and activation of various sperm transcription factors (Aitken and Baker, 2006; Sabeti et al., 2016; Aitken, 2017). Our review suggests that older males have lower antioxidant levels but higher oxidative stress markers in their seminal fluid, and thus may have higher oxidative stress than young males. The decline in antioxidants might indicate a tradeoff where ageing males cannot maintain optimal antioxidant levels if these are energetically costly.

The mechanisms for why older males have higher oxidative stress could be several. For instance, ROS from sperm could "leak" into seminal fluid or somatic cells which produce SF could accumulate more ROS damage over time in old versus young males. This increase in oxidative stress in older males could have severe hypothesized functional consequences, such as higher oxidative damage to sperm, or the fertilized egg, and reduced sperm performance, which can be tested by future studies. More studies are needed to disentangle the origin/cause of age-dependent changes in ROS production in seminal fluid, the consequences of scavenging by SF antioxidants, and the overall effects on sperm, male and female reproduction.

## 3.5. Factors that could influence seminal fluid ageing rates

Studies identified in our systematic literature review included only a few factors such as proportional lifespan sampled, male mating history and sampling of males to explain differences in seminal fluid ageing.

Besides these, other factors could be predicted to influence SF ageing. For instance, evidence for reproductive ageing has been shown to be stronger in laboratory and captive animals compared to wild ones (Nussey et al., 2013; Zajitschek et al., 2020; Kappeler et al., 2022). Additionally, domestic animals, which were used in a majority of studies found in our systematic review, are kept in semi-controlled conditions and are killed off prior to reaching a senescent age (i.e., post their "prime"). Thus, evidence for reproductive senescence in the seminal fluid may be weaker in domestic animals, although in our review, we found evidence for seminal fluid senescence in both lab and domestic animals.

Other abiotic and biotic factors could influence seminal fluid ageing, such as a male's social environment. Both sperm and seminal fluid are highly plastic in their expression (reviewed in Perry et al., 2013; and Ramm, 2020). Males are known to invest more in seminal fluid production under more competitive environments, such as under high sperm competition (Hopkins et al., 2019b), possibly at the cost of reduced later-life investment in reproduction (Lemaître et al., 2020a). The costs of ejaculate plasticity have been discussed before (see, e.g., Ramm, 2020), but to our knowledge have not been tested, and whether these costs differ for old versus young males can

be investigated in the future. Knowledge of costs could be one factor predicting ageing trajectories of seminal fluid. If seminal fluid production is costly and its continued production causes damage, then strong selection on early reproduction might be favoured and we would expect rapid ageing as a consequence. However, if seminal fluid production is cheap then factors such a sperm competition, male dominance and/ or female preferences might have more scope to shape ageing patterns. For example if old males are socially dominant and preferred by females they might face little competition and selection on seminal fluid is relaxed and thus ageing might arise. Conversely, if older males are more likely to experience sperm competition or female ejaculate rejection then there might be relatively high selection for seminal fluid competency late in life. Additionally, sperm production patterns, i.e., continuously versus one bout early in life, and whether sperm is the limiting factor might be important too, because supply and demand needs to be balanced between these different components through reproductive lifespan. To test these ideas knowledge of ejaculate ageing across a broad range of taxa with different reproductive patterns is necessary.

Mating systems can also influence ageing of seminal fluid. Ageing effects are expected to be more pronounced in polyandrous species where males are likely to invest more in their ejaculates (Veltsos et al., 2022), due to facing a higher risk of sperm competition. However, the influence of sperm competition on male reproductive senescence likely depends on the life-history of a species. For example, in some species, males may preferentially invest resources in producing more SFPs early in life, and suffer faster rates of reproductive senescence later in life (see also Lemaître et al., 2020a). Older males in such species are often inferior in both pre- and postcopulatory competition (Johnson and Gemmell, 2012; Gasparini et al., 2019) and are discriminated against by females (Velando et al., 2011; Rezaei et al., 2015). Alternatively, species with increased levels of sperm competition may evolve increased investment in SF (Immler et al., 2011; Lüpold et al., 2020), which may reduce the rate of senescence in these ejaculate traits (Delbarco-Trillo et al., 2018).

Abiotic factors such as nutrition could also impact the trajectory of ageing of the seminal fluid. Studies on male rats showed that both over- and undernutrition during pregnancy seem to lead to premature male reproductive ageing (reviewed in Zambrano et al., 2021). This is because, at least in mammals, the early stages of development have an overall impact on health and quality of life during adulthood (developmental programming) with endocrine disruptors and maternal nutrition impacting developmental programming.

### 3.6. Male ageing effects on offspring fitness *via* seminal fluid

The impact of male age is not limited to his own and his mates' reproductive success, but potentially extends to offspring fitness as well. Males are known to influence the fitness of their offspring through mechanisms other than the transmission of DNA (Curley et al., 2011; Crean and Bonduriansky, 2014). Advanced paternal age has been shown to shorten offspring lifespan, exacerbate ageing-related pathology and to alter offspring social behavior (Kong et al., 2012; Brenman-Suttner et al., 2018; Xie et al., 2018). Classically, these impacts were believed to be due to the accumulation of *de novo* mutations in ageing germ cells. However, recent work suggests that non-genetic mechanisms, such as changes in methylation patterns or small non-coding RNA populations, are more likely to drive the intergenerational effects of ageing (Xie et al., 2018). Importantly, it was recently suggested that seminal fluid might be an under-appreciated mediator of paternal effects (Simmons, 2011; Watkins et al., 2018; Evans et al., 2019; Simmons and Lovegrove, 2019, 2020; Kekäläinen et al., 2020), yet this has not yet been tested in a paternal ageing context.

## 4. Conclusion

Despite the low number of studies found, our review is crucial in highlighting the gaps in our knowledge of seminal fluid ageing. Our review generates hypotheses on how ageing of seminal fluid could affect male and female fitness, and makes predictions for how various biological and methodological factors could modulate the effects of seminal fluid ageing. It further shows that ageing impacts the level of oxidative stress in the seminal fluid, and to some extent the abundance of SFPs in the ejaculate. We highlight how the age-dependent changes observed in the seminal fluid profile can affect male fitness. Additionally, we find that male ageing can alter expression or abundance of specific SFPs that regulate female post-mating behavior (Koppik and Fricke, 2017; Sepil et al., 2020), oviposition rate (Marshall et al., 2009), male sperm competition (Ruhmann et al., 2018; Sepil et al., 2020), response to oxidative stress (Kant et al., 2019; Westfalewicz et al., 2021), immune and antimicrobial function (Borziak et al., 2016), and sperm velocity (Borziak et al., 2016). Most research to date has been done on mammals and insects, specifically on species important for animal husbandry or biomedicine. Hence, broadening the taxonomic spread of future studies in general, and the inclusion of species with different mating systems in particular should be a priority.

We highlight how understanding reproductive ageing of sperm, but also of the seminal fluid and the tissues producing them can provide a better picture of male reproductive ageing. Any future research agenda must therefore include a more focused assessment of the downstream consequences of seminal fluid ageing on fitness-related traits, encompassing impacts on fertility, sperm competitive ability and effects on the resulting offspring. Future work should ideally study the non-sperm ejaculate components as a whole, together with changes in sperm as this will be key to advance our understanding of male reproductive ageing.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1066022/full#supplementary-material

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EDITED BY
Rita V. M. Rio,
West Virginia University, United States

REVIEWED BY
Jorge Contreras-Garduño,
National Autonomous University of Mexico, Mexico
Venkatesh Nagarajan Radha,
The University of Sydney, Australia

*CORRESPONDENCE
Sinead English
✉ sinead.english@bristol.ac.uk

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# Investigating the unaccounted ones: insights on age-dependent reproductive loss in a viviparous fly

Sinead English[1]*, Antoine M. G. Barreaux[1,2,3], Robert Leyland[4], Jennifer S. Lord[4], John W. Hargrove[5], Glyn A. Vale[6] and Lee R. Haines[4,7]

[1]School of Biological Sciences, University of Bristol, Bristol, United Kingdom, [2]Intertryp, Université Montpellier, CIRAD, IRD, Montpellier, France, [3]Animal Health Theme, ICIPE, Nairobi, Kenya, [4]Liverpool School of Tropical Medicine, Liverpool, United Kingdom, [5]South African Centre for Epidemiological Modelling and Analysis, Stellenbosch University, Stellenbosch, South Africa, [6]National Resources Institute, University of Greenwich, Chatham, United Kingdom, [7]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, United States

Most empirical and theoretical studies on reproductive senescence focus on observable attributes of offspring produced, such as size or postnatal survival. While harder to study, an important outcome of reproduction for a breeding individual is whether a viable offspring is produced at all. While prenatal mortality can sometimes be directly observed, this can also be indicated through an increase in the interval between offspring production. Both direct reproductive loss and presumed losses have been found to increase in older females across several species. Here, we study such reproductive loss (or "abortion") in tsetse, a viviparous and relatively long-lived fly with high maternal allocation. We consider how age-dependent patterns of abortion depend on the developmental stage of offspring and find that, as per previous laboratory studies, older females have higher rates of abortion at the late-larval stage, while egg-stage abortions are high both for very young and older females. We track the reproductive output of individual females and find little evidence that experiencing an abortion is an adaptive strategy to improve future reproductive outcomes. After an abortion, females do not generally take less time to produce their next offspring, these offspring are not larger, and there is no sex bias towards females, the sex with presumed higher fitness returns (being slightly larger and longer-lived than males, and with high insemination rates). Abortion rates are higher for breeding females experiencing stress, measured as nutritional deprivation, which echoes previous work in tsetse and other viviparous species, i.e., humans and baboons. We discuss our results in the context of studies on reproductive loss across taxa and argue that this is an important yet often overlooked reproductive trait which can vary with maternal age and can also depend on environmental stressors.

KEYWORDS

**tsetse, pregnancy loss, prenatal mortality, reproductive senescence, offspring quality**

## 1. Introduction

Older breeders tend to have reduced reproductive performance, producing smaller or poorer quality offspring with shorter lifespans (Monaghan et al., 2008, 2020; Ivimey-Cook and Moorad, 2020). There is extensive empirical evidence for such reproductive senescence across taxa, both in the wild (Berman et al., 2009; Nussey et al., 2009; Sharp and Clutton-Brock, 2010; Lemaître and Gaillard, 2017) and laboratory (Miller et al., 2014; de Boer et al., 2018; Lord et al., 2021). Many studies also find that younger breeders have lower reproductive output (e.g.,

Zedrosser et al., 2009; Lord et al., 2021). This results in a bell-shaped (i.e., inverse U-shaped) pattern of offspring size or quality with parental age. Our focus here is on species where females carry the main costs of reproduction; accordingly, hereafter, we consider specifically the effect of maternal age.

Several theoretical models have investigated why bell-shaped, age-dependent allocation might be optimal. In terms of the later-life decline, there may be antagonistic links between traits that favour early survival or reproduction even if these come at a cost of later-life output (Kirkwood, 1977), particularly as the strength of selection declines with age. Moreover, given that reproduction can result in physiological damage, older females may strategically reduce investment in reproduction to reduce damage-induced mortality (McNamara et al., 2009). The bell-shaped curve may be associated with extrinsic mortality, such as predation (Cichoń, 2001), or age-specific trends in energy intake or the physiological costs of reproduction (Barreaux et al., 2022).

While reproductive output tends to be measured in terms of offspring quality (size or physiological condition), one might also predict age-dependent effects on prenatal mortality or early reproductive failures (termed "spontaneous abortion" or "abortion" hereafter). There are several reasons for spontaneous abortion to be higher at certain ages, in particular for very young or old mothers, based on theories about reproductive senescence (e.g., Kirkwood, 1977; McNamara et al., 2009). First, where there are trade-offs between investing in offspring production versus self-maintenance, breeding females may favour self-preservation when they are younger, and have a longer potential reproductive lifespan ahead, even if this comes at a risk of higher offspring mortality (the "adaptive" hypothesis). Second, the risk of spontaneous abortion may reflect physiological constraints, for example on the amount of energy available to invest in offspring, which could be lower in young, inexperienced mothers (the "constraint" hypothesis). Related to this, as females get older, they could accumulate physiological damage, from cellular processes (such as damage associated with reactive oxygen species) through to organ or other anatomical deterioration, and thus older females could be in compromised physiological condition to produce viable offspring. Both adaptive and constraint hypotheses have implications for whether abortions happen early or later during the pregnancy period, i.e., depending on how much has already been invested; and for whether abortions are more likely to occur for a particular sex, depending on sex differences in fitness returns, or in the energetic requirements of male or female offspring.

Comparative data on age-specific abortions across animals are rare, owing to difficulties in observing pre-natal mortality (Graham, 1979). There is considerable evidence across human populations that rates of pre-natal mortality, termed miscarriage, increase with maternal age at conception (Forbes, 1997; Nybo Andersen et al., 2000; Brosens et al., 2022; Zhang et al., 2022), are slightly higher in young mothers (Brosens et al., 2022), and miscarriages are more prevalent in the first trimester of pregnancy (Ammon Avalos et al., 2012; Brosens et al., 2022). Rather than focusing on age-specific effects, most studies in non-human species find that spontaneous abortions increase with maternal stress (Young et al., 2006; Cant et al., 2010), or occur when pregnant females are exposed to an unfamiliar male (Bruce, 1960; Roberts et al., 2012). A recent study in wild baboons has shown, however, that both age and ecological factors affect pregnancy failures (Fogel et al., 2023): younger and older females have higher rates of foetal loss, as do females experiencing heat stress or poor habitats. Studies in dolphins and macaques show that interbirth intervals increase with maternal age (Paul et al., 1993; Karniski et al., 2018), which could be indicative of an increased risk of spontaneous abortion. As these species also exhibit postnatal care, however, such intervals may instead be due to more prolonged care and lower conception rates among older females.

Here, we investigate age-dependent patterns of abortion in live-bearing tsetse (*Glossina* spp.). Female tsetse give birth to a single larva, which, after developing through three instar stages *in utero*, is almost the same weight as the mother (Benoit et al., 2015; Haines et al., 2020). Furthermore, after birth, the female immediately starts the next reproductive cycle using stored sperm and is thus in an almost continuous state of pregnancy (Denlinger and Ma, 1974). Because of this unique life history, female tsetse are excellent models for studying reproductive senescence due to their high maternal investment, iteroparous reproduction and relatively long lifespans, living up to 8 months in the field (Carpenter, 1913). Moreover, as tsetse females do not provide postnatal care, patterns of interbirth interval reflect *in utero* constraints or strategies and are not confounded by subsequent investment.

One might expect abortion rates to increase with age in tsetse due to reproductive senescence, yet mixed evidence is reported in the literature. Laboratory studies indicate that the probability of spontaneous larval abortion increases with female age (Lord et al., 2021). Estimated abortion rates in field populations are low, however, and do not increase with age (Hargrove, 1999). On the contrary, if empty uteri can be taken as evidence for a recent abortion, it seems that in the field young females have relatively high abortion rates (Hargrove, 1999), and that the rates are not high in older flies (Hargrove, 2023). Physiological and environmental stress are stronger predictors of overall abortion than age in tsetse, in both field and laboratory studies. Abortion rates are elevated for females in low nutritional condition (Mellanby, 1937; Saunders, 1972; Lord et al., 2021) and for tsetse exposed to insecticides (Riordan, 1986). While abortions are infrequent in wild tsetse, they do increase during hotter months of the year (Hargrove, 1999, 2023), when flies exhibit increased mortality levels (Hargrove, 2001) and are likely experiencing more physiological stress, due for example to host scarcity or thermal costs of mobility.

Existing studies of patterns of abortion in tsetse have two main gaps. First, studies have not distinguished between abortions in the early and late stages of pregnancy, and egg-stage abortions are often undetected as they are difficult to visualise in the substrate (i.e., leaf litter) and are too small to detect without microscopy. Such distinctions are important, however, when testing adaptive hypotheses because any optimal decision about terminating a reproductive event may depend on how much a female has already invested. In tsetse, the majority of larval growth happens in the final stage of pregnancy (Denlinger and Ma, 1974; Tobe and Langley, 1978; Hargrove and Muzari, 2015) so earlier-stage abortions could reflect more of an adaptive strategy, before females have made any significant investment in their offspring. Second, it is not known how abortion affects subsequent reproduction, which requires tracking the reproductive output of individual females. If females strategically abort offspring to reduce resource loss, they may take less time to produce their next offspring (Saunders, 1960). Alternatively, if abortion is an indication of female stress and this carries over to the next reproductive event, females may take longer to produce their

next offspring, and these offspring might also weigh less. Predictions concerning the relationship between abortions and offspring sex are more nuanced, but we hypothesize that females might strategically abort male offspring when they lack sufficient resources to produce enough of both sexes—either as they get older, or due to nutritional stress. This is based on our assumption that females might provide higher fitness returns. The assumption arose from three considerations. First, females are larger (as indicated by wing length) at maturity (Hargrove et al., 2019), even though female and male pupae are similar in weight. Second, females are longer lived (Hargrove et al., 2011). Third, insemination rates in the field are high (90%, Hargrove, 2012). Thus, while we do not know the sex of aborted offspring, there could be a potential bias towards producing female offspring immediately after an abortion if mothers that are old or in poor conditions are likely to abort males.

Here, we investigate patterns of age-specific abortion in tsetse using data from a laboratory-reared tsetse colony. First, we measure stage-specific abortions in females of known age and, second, we track the reproductive history of individual females. We ask whether egg-stage abortions increase with age as observed in previous studies for later, larval-stage abortions, and whether they are also higher in young females as found in field studies. We then use individual-level data to address the question of how an abortion affects the size and sex of the next offspring and the period required to produce it. This leads to potential insights on whether patterns of abortion, according to female age or condition, are due to strategic holding back of female resources or a lack of ability to invest in a particular reproductive event.

## 2. Materials and methods

We analysed the causes and consequences of spontaneous abortions using known-age tsetse, *Glossina morsitans morsitans* Westwood, maintained at the Liverpool School of Tropical Medicine (LSTM). As described in Files S1 and S2 by Lord et al. (2021), the LSTM tsetse colony comprises multiple breeding cages, each with 48 females and 12 males. Each week, 12 new cages are made and placed on a tray (each totaling, initially, 576 females and 144 males) and maintained for up to 3 months. Every week, a new tray is created and the oldest discarded, thus each tray contains age-matched females; and reproductive output of each tray is monitored three times weekly. Unless specified otherwise below, flies were fed defibrinated horse blood (TCS Biosciences Ltd) and maintained at 26°C (±2°C), 72 (±4) % humidity and a photoperiod of 12 h.

Below, we describe two studies conducted on flies from the LSTM colony to understand the causes and consequences of age-specific abortions in female tsetse, one which involves a detailed analysis of stage-specific (i.e., egg and larval) abortions of colony flies ("study 1") and the other, which involves an experiment that manipulated nutrition in individually-housed flies [as described in Lord et al. (2021), here termed "study 2"]. All statistical analyses were conducted in R version 4.2.1 (R Core Team, 2022) using RStudio version 2022.7.1.554 (RStudio Team, 2022).

### 2.1. Study 1: changes in abortions with maternal age and stage of larval development

First, we conducted analyses on changes in abortion by female age and offspring stage at the colony-level. Previous insights on tsetse abortions have focused on larval stages visible without magnification (e.g., Lord et al., 2021). Here, we used a more detailed approach to quantify abortions from the egg stage for females of known age in the LSTM tsetse colony. In total, we measured the reproductive output of 35 trays, across four time periods (22–29 November 2021 [period 1, $n = 7$ trays]; 21 February 2022 to 6 March 2022 [period 2, $n = 8$ trays]; 7–22 March 2022 [period 3, $n = 10$ trays]; and 23 March 2022 to 8 April 2022 [period 4, $n = 10$ trays]).

During each period, we checked the colony trays three times per week to record viable births (numbers of pupae deposited and total pupal mass), as well as abortions. For the latter, we collected material from below the colony cages and, using a dissecting microscope (40X), examined the "tsetse dirt" (Figure 1A), which is a collection of frass, spermatophores, aborted eggs and larvae to detect aborted offspring of various developmental stages. These were categorised as: (1) Egg: smooth, intact pearlescent egg with a defined pellicle, (2) L1: larva is small, opaque and pale yellow, with no visible polypneustic lobes, (3) L2: larva is opaque, darker yellow, polypneustic lobes are formed but not melanised, and (4) L3: black polypneustic lobes are visible and larval segmentation is evident (Figures 1B–D). Counts were made per-tray. For periods 1 and 2, the maximum ages of females recorded were 9 and 10 weeks respectively, whereas for periods 3 and 4 abortion data were available for females up to the age of 12 weeks. Note that <10% of females die before the age of 12 weeks (Haines et al., unpublished data).

We analysed how stage-specific abortions vary across maternal age by fitting a generalised additive model to the data to capture any non-linear patterns, using the R package "mgcv" (Wood, 2017). We fit GAMs with knots (i.e., points at which the slope changes) varying from 3 to 10 and compared models using Akaike Information Criteria, corrected for small sample sizes (AICc). Plots were made using the GAM for each stage with the lowest AICc.

### 2.2. Study 2: (i) estimating presumed abortions in individually tracked females and (ii) quantifying effect of known or presumed previous abortion on subsequent larviposition duration, offspring weight and offspring sex

We conducted an experiment on individually-caged females, originating from the same colony, to establish the effect of nutritional stress and mating history on age-dependent reproductive allocation [see detailed methods in Lord et al. (2021)]. Previously, we considered the overall effects of reduced nutrition and delayed mating on offspring quality and survival across female age. Here, we investigate individual-level consequences of abortion for subsequent reproduction; and any potential bias towards producing a particular sex in older females or following an abortion. We focus on the contrast between nutritionally stressed females and those fed the rearing standard defibrinated horse blood ("control"). Nutritionally stressed

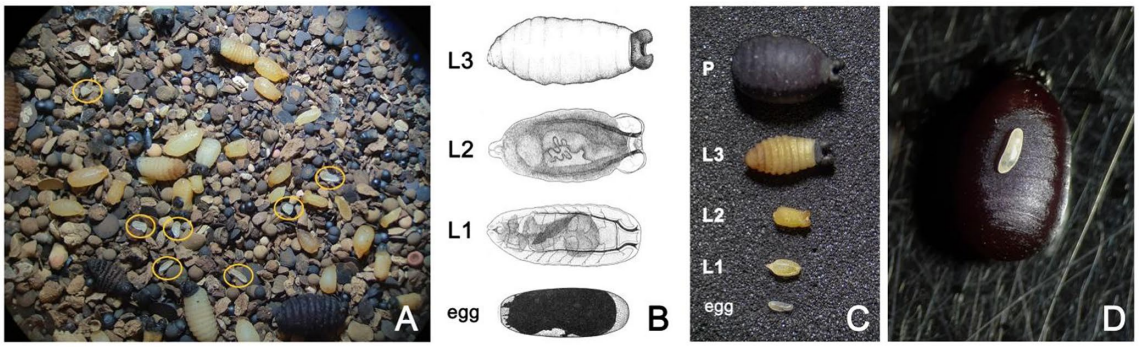


FIGURE 1
Isolation and characterisation of different stages of tsetse development. **(A)** Petri dish containing material (tsetse dirt) collected from a tray of tsetse aged 3weeks containing fly frass, and premature larval and egg (seven yellow circles) depositions. **(B)** Detailed diagrams of each gestational stage [modified from Burtt and Jackson (1951)]: from egg to mature 3rd instar larva. **(C)** The reproductive losses collected from each tray were scored as: egg, L1: first instar larva lacking polypneustic lobes, L2: second instar larva with distinct polypneustic lobes, L3: third instar larva with distinct body segmentation and black polypneustic lobes, P: full term pupated larva (pupa). **(D)** Size comparison between an egg and the extraordinary exponential growth it undergoes in 4–5days to become a pupa.

females were provided a bloodmeal adjusted with serum to have a lower packed cell volume (hematocrit, PCV)—10% PCV versus a control of 45% PCV—to mimic an animal sick with anaemia-related trypanosomiasis (Mamoudou et al., 2015).

Previous studies on laboratory and field tsetse have found that the inter-larval period follows a consistent timing for flies kept at constant temperature, being, on average, 16 days to produce the first larva and 9 days for subsequent larvae at 25°C (Mellanby, 1937; Denlinger and Ma, 1974; Hargrove, 2004). However, when we tracked reproductive output of individual females ($n = 190$) mated within 48 h of emergence, the inter-larval period was often considerably longer than expected—even when known larval abortions were excluded, being up to 52 days to produce the first larva and up to 32 days between subsequent larvae. We postulated that such variability may be due to the presence of egg-stage, or very early larval-stage, abortions which were not recorded in this experiment. Henceforth, we applied the Hampel filter (Pearson, 2002) to our dataset to establish an unbiased threshold for detecting such assumed egg abortions. This works by deeming as outliers any data points 3 or more median absolute deviations (MAD) from the median.

We analysed whether such presumed abortions are higher in nutritionally stressed females, and increase with female age, in line with our previous findings regarding confirmed abortions (Lord et al., 2021). For this analysis, we excluded known abortions, and coded each gestation interval as having produced offspring within a normal interval (0), or having taken too long (1, hence likely including an undetected abortion based on the threshold as described above). We then conducted a binomial GLM, on $n = 93$ control flies and $n = 86$ nutritionally stressed flies, including as covariates experimental treatment, and linear and quadratic maternal age effects. Maternal age was $z$-transformed. We established the significance of input variables by inspecting the effect size and standard error for each term included in the main model.

We were also interested in whether females that aborted an offspring could then accelerate the time to produce their next larva, and what the consequences of a previous abortion were for the size and sex of subsequent offspring. We thus compared the larviposition duration, pupal wet weight (in mg) and offspring sex after a known viable birth to those produced after a recorded abortion, or after a presumed abortion. For this analysis, we included $n = 91$ control flies and $n = 77$ nutritionally stressed flies.

We analysed the effect of previous birth outcome on subsequent birth outcome using linear mixed-effect models (LMM), for larviposition duration and offspring weight, and general linear mixed-effect models (GLMM), for offspring sex, using the R library lme4 (Bates et al., 2015). We considered only those larvipositions where a viable pupa was produced, within the accepted thresholds for a larviposition (i.e., 8–14 days). For each model, we included previous birth outcome (no abortion, confirmed abortion or presumed abortion in previous inter-larval period) as an input variable, and accounted for maternal treatment (nutritional stress or control) and $z$-transformed maternal age (linear and quadratic) as potential covariates. We also included interactions between previous birth outcome and treatment to ask whether any carryover effects from previous larvipositions depend on maternal nutritional stress. Maternal identity was included as a random effect to account for repeated measures on individual mothers. As above, significance of input variables was established by inspecting the effect size and standard error for each term included in the main model, and by conducting ANOVA comparisons of models.

## 3. Results

### 3.1. Study 1: changes in abortions with maternal age and stage of larval development

When we consider stage-specific abortions, we find that egg abortions are relatively more frequent in younger females (when larval abortions are rare) and again in older flies (Figure 2), whereas larval-stage abortions (L1, L2, or L3) are rare in younger females and increase in frequency as females get older. Overall, there was a trend for there to be higher egg-stage abortions than abortions at later larval stages (Figure 2)—particularly for very young and older females—however, due to low sample sizes, formal statistical comparison was not possible.

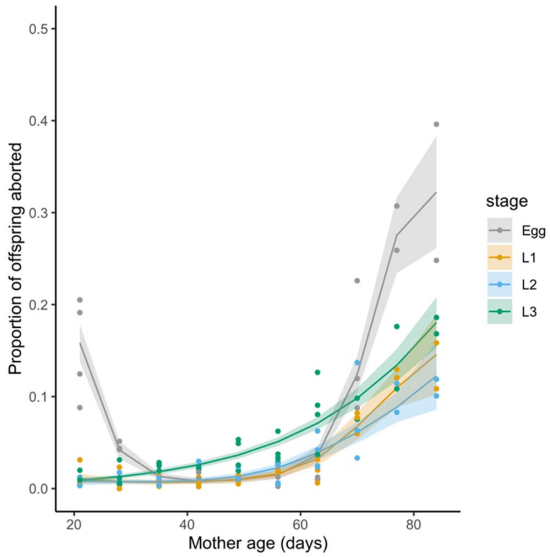


FIGURE 2
Abortions, as a proportion of total pregnancies (i.e., pupae plus abortions) and as a function of female age in days. Different colours indicate the different developmental stages considered (egg, L1, L2, or L3 larvae). Individual points represent data for each collection period, lines and shading indicate predictions and 2×standard error from a GAM fitted to each developmental stage.

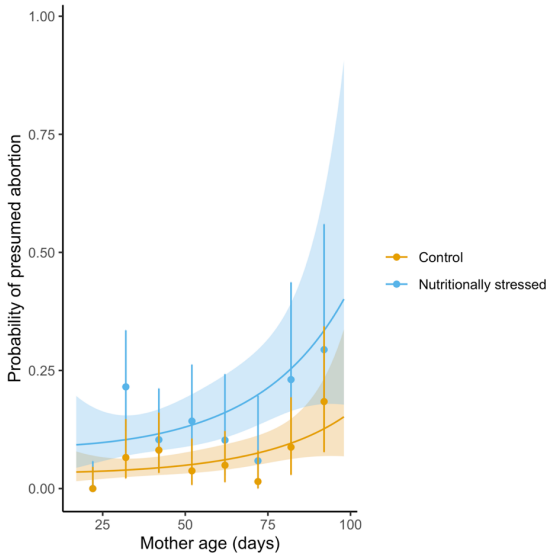


FIGURE 3
Probability of a presumed abortion per inter-larval period—based on being longer than the expected, typical period—as a function of maternal diet (blue=nutritionally stressed, 10% PCV blood treatment, orange=control 45% PCV blood treatment) and age. Lines denote predicted probabilities based on a GLMM fit to the data, with 95% prediction intervals (shaded area); and points are the means for 10-day periods with exact binomial 95% confidence intervals.

## 3.2. Study 2: (i) estimating presumed abortions in individually tracked females and (ii) quantifying effect of known or presumed previous abortion on subsequent larviposition duration, offspring weight and offspring sex

Applying the Hampel filter to our experimental data, we established 16–28 days as an interval for females to produce their first larva without any undetected abortion; and 8–14 days between subsequent larvae. We accept that these intervals are wider than reported inter-larval periods at constant temperature, but we use these thresholds as a more conservative and unbiased approach based on data distributions.

We found that, as shown in our previous work on confirmed larval abortions, the incidence of presumed egg or larval abortions—based on the inter-larval period being above the threshold of 28 days (first larva) or 14 days (subsequent larvae)—is higher in females who experienced nutritional stress (effect of treatment: 0.971 ±0.246, $z = 3.94$, $p < 0.0001$, Figure 3). We did not, however, find any significant association in the likelihood of a presumed abortion and maternal age, with both linear and quadratic terms of maternal age having small effect sizes (0.00052 and 0.00016, $p = 0.986$ and 0.23 respectively).

We considered whether having an abortion (either confirmed or presumed) affected the time taken to produce the next viable offspring, its mass, or its sex (Table 1). We found that experiencing an abortion did influence time taken to produce the next offspring, although this depended on maternal experimental treatment (effect of previous outcome × treatment: $F_{2,690} = 14.33$, $p < 0.0001$, Figure 4A). Control females, raised on a diet of undiluted blood, took on average 1.02 days longer to produce their next offspring after aborting a previous one (when such an abortion was observed rather than presumed) compared to when they produced a viable offspring. In contrast, nutritionally stressed females, fed diluted blood, took slightly less time (0.68 days) to produce their next offspring after a confirmed abortion. Maternal age (as linear or quadratic term) did not affect the time taken to produce the next viable offspring (Table 1).

When we considered the effect of previous birth outcome on subsequent offspring mass, we found, again, that any effect depended on maternal experimental treatment (effect of previous outcome × treatment: $F_{2,666} = 4.54$, $p = 0.011$, Table 2; Figure 4B). Specifically, females on the control diet produced smaller offspring after having an observed abortion than after a normal birth or presumed abortion; whereas there was no difference among nutritionally stressed females in the size of offspring produced depending on previous birth outcome. As shown previously, we found both the linear and quadratic term for maternal age to be significant in the model (Table 2).

We found no effect of previous birth outcome on whether the next offspring produced was a male or female ($\chi^2 = 2.74$, $p = 0.254$), nor was the probability of a sex bias affected by maternal age or experimental treatment (Table 3).

# 4. Discussion

We used detailed observations on stage-specific abortions, and experimental data tracking individual reproductive output, to investigate age-dependent patterns of early-reproductive failure in laboratory tsetse. We found that, in contrast to previous work—where larval abortions increase with age in females—egg-stage abortions are also elevated in younger females. Taken together, these abortion

TABLE 1 Full model results for linear mixed model (LMM) on time taken to produce next viable offspring depending on previous birth outcome.

| | Estimate | SE | df | *t* value | *P*-value |
|---|---|---|---|---|---|
| Intercept | 10.83 | 0.07 | 218.84 | 155.20 | <0.0001 |
| Previous abortion (confirmed) | 1.00 | 0.25 | 690.60 | 4.04 | <0.0001 |
| Previous abortion (presumed) | 0.07 | 0.23 | 690.48 | 0.28 | 0.779 |
| Experimental treatment (nutritional stress) | 0.15 | 0.10 | 190.41 | 1.46 | 0.147 |
| Maternal age (*z*-transformed) | 0.01 | 0.04 | 632.41 | 0.24 | 0.814 |
| Maternal age$^2$ (*z*-transformed) | −0.01 | 0.03 | 608.53 | −0.33 | 0.742 |
| Previous outcome (confirmed) × Treatment (nutrition) | −1.72 | 0.32 | 690.92 | −5.31 | <0.0001 |
| Previous outcome (presumed) × Treatment (nutrition) | 0.10 | 0.31 | 689.23 | 0.32 | 0.752 |

Model based on $n = 699$ observations of 168 females (random effect variance ± SD: 0.165 ± 0.406).

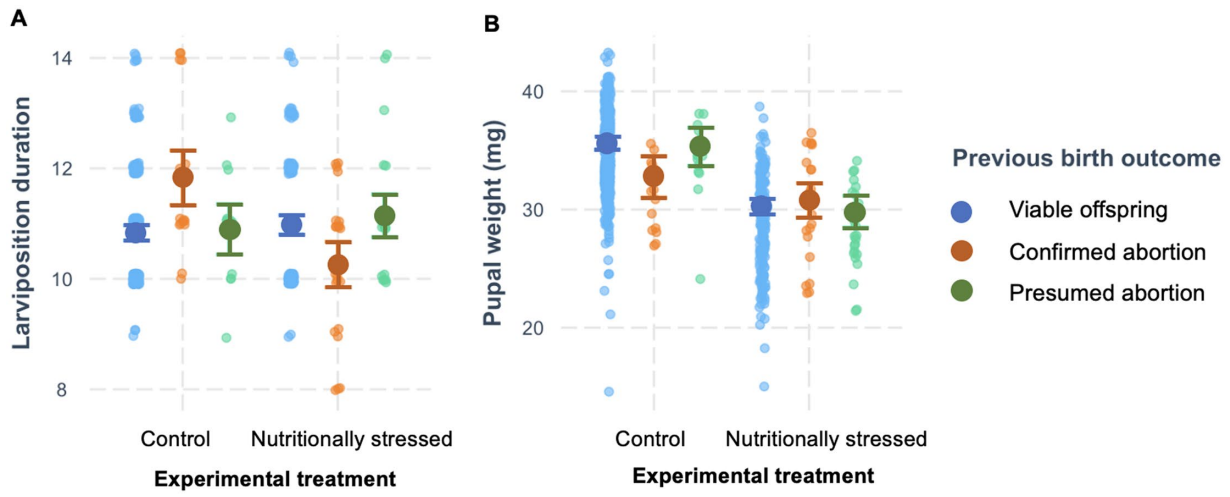


FIGURE 4
Effect of previous birth outcome on **(A)** subsequent larviposition duration and **(B)** subsequent pupal weight. Shown are the raw data (points), and predictions with 95% prediction intervals (generated using the cat_plot function in R library "interactions", Long, 2019) from the LMM on larviposition duration and pupal weight, respectively, depending on maternal treatment (control or nutritionally stressed).

patterns follow a similar U-shaped (or inverse-bell-shaped) curve consistent with studies on foetal loss in humans and baboons, and which would fit both adaptive- and constraint-based predictions about age-dependent changes in reproductive loss. We then analysed individual data to investigate the consequences of experiencing an abortion for subsequent reproductive outcomes. We found that there is no clear evidence that abortion is an adaptive strategy to improve future reproductive success; after an abortion, females do not generally take less time to produce their next offspring, these offspring are not larger, and they do not produce more females (the sex with higher presumed fitness returns, living longer and being more likely to produce offspring consistently when mated).

Our study is the first to quantify, using detailed observation of the debris below tsetse breeding cages, how egg-stage abortions change across female age. Previous studies have focused on larval-stage abortions—which are observable without inspection under a microscope—and have shown that, in the laboratory, such abortions increase as females get older (Lord et al., 2021). In general, we find a relatively high number of egg-stage abortions compared to later stage abortions, which indicates that these are likely under-represented in other studies. We also find that egg-stage abortions increase as females get older, but these are also relatively high in younger females. Thus, the U-shaped pattern of abortion fits with our other work—and more general observations of age-dependent reproductive output (Monaghan et al., 2020)—that both younger and older females have compromised reproduction (smaller offspring, higher pre-natal mortality). Higher rates of reproductive loss in younger females also align with field studies in tsetse, which show that younger females tend to produce smaller offspring (English et al., 2016; Hargrove et al., 2018) and have a higher proportion of empty uteri, which is indicative of a recent abortion (Hargrove, 1999). Females producing their first offspring may not be in physiologically prime condition (Haines, 2013); early in the first pregnancy they may have lower levels of fat thoracic musculature than fully mature flies and so have less aptitude at host searching (Hargrove, 1975). Moreover, they may have lower fat stores and thus be unable to provide sufficient milk for a successful pregnancy. Given that they are at the onset of their reproductive career, terminating reproductive events early in gestation may be an adaptive strategy to retain resources for their own maintenance and future reproduction. We are not aware of any theoretical models on maternal allocation with offspring prenatal mortality across different developmental stages as an outcome: future work investigating

TABLE 2 Full model results for linear mixed model (LMM) on weight of next viable offspring depending on previous birth outcome.

| | Estimate | SE | df | *t* value | *P*-value |
|---|---|---|---|---|---|
| Intercept | 35.66 | 0.27 | 188.41 | 130.38 | <0.0001 |
| Previous abortion (confirmed) | −2.87 | 0.88 | 669.21 | −3.27 | 0.001 |
| Previous abortion (presumed) | −0.33 | 0.83 | 670.98 | −0.40 | 0.688 |
| Experimental treatment (nutritional stress) | −5.40 | 0.40 | 176.59 | −13.40 | <0.0001 |
| Maternal age (*z*-transformed) | 0.97 | 0.12 | 593.70 | 7.80 | <0.0001 |
| Maternal age$^2$ (*z*-transformed) | −1.16 | 0.12 | 562.96 | −10.00 | <0.0001 |
| Previous outcome (confirmed) × Treatment (nutrition) | 3.41 | 1.14 | 667.90 | 3.00 | 0.003 |
| Previous outcome (presumed) × Treatment (nutrition) | −0.10 | 1.07 | 665.41 | −0.09 | 0.927 |

Model based on $n = 682$ observations of 168 females (random effect variance ± SD: 3.702 ± 1.924).

TABLE 3 Full model results for generalised linear mixed model (GLMM) on sex of next viable offspring depending on previous birth outcome.

| | Estimate | SE | *z* value | *P*-value |
|---|---|---|---|---|
| Intercept | 0.02 | 0.17 | 0.11 | 0.915 |
| Previous abortion (confirmed) | 0.21 | 0.66 | 0.32 | 0.747 |
| Previous abortion (presumed) | −0.57 | 0.77 | −0.73 | 0.464 |
| Experimental treatment (nutritional stress) | 0.00 | 0.25 | 0.02 | 0.985 |
| Maternal age (*z*-transformed) | −0.21 | 0.11 | −1.87 | 0.061 |
| Maternal age$^2$ (*z*-transformed) | 0.09 | 0.10 | 0.92 | 0.36 |
| Previous outcome (confirmed) × Treatment (nutrition) | 0.04 | 0.91 | 0.04 | 0.969 |
| Previous outcome (presumed) × Treatment (nutrition) | −0.27 | 0.98 | −0.28 | 0.779 |

Model based on $n = 413$ observations of 156 females (random effect variance ± SD: 0.148 ± 0.385).

different adaptive strategies of withholding allocation early or late in pregnancy depending on female age and physiological condition will yield predictions for empirical studies.

Our analyses both on confirmed and presumed pregnancy losses (based on longer than typical inter-larval period) highlight the strong effect of female nutritional stress on elevated abortion rates across all ages in tsetse. Such a link between stress and abortion has been shown previously in tsetse, when considering stress in terms of nutritional deprivation (Mellanby, 1937; Saunders, 1972; Lord et al., 2021), exposure to insecticides or toxicants in bloodmeals (Saunders, 1971; Riordan, 1986) and, in field studies, exposure to heat stress (Hargrove, 1999). These findings resonate with a recent study on wild hybrid baboons which showed that foetal loss is higher when females experience high temperatures late in pregnancy, and when they occupy a poor-quality habitat (Fogel et al., 2023). There is increasing appreciation that climate change and extreme heat will affect not just survival but fertility across a range of species (Walsh et al., 2019), including humans, but owing to difficulty in measuring prenatal loss or reproductive failure, fewer studies focus on this as an outcome. Modelling of tsetse populations tends to focus on adult mortality and pupal emergence rates, which are impacted by host availability and increasing maximum temperatures (Lord et al., 2018). Findings from this study, and related field studies, highlight how such environmental stress also affects prenatal mortality and should thus be considered in future modeling work to predict tsetse population responses to climate change and the emergent disease dynamics.

We acknowledge that our findings are limited to the laboratory context and caution should thus be applied when generalising to field conditions. In the laboratory, tsetse mortality rates are lower as there is no predation and flies are offered regular and predictable access to blood. Laboratory females do not have the opportunity to fly around freely, and they thus accumulate fat as they get older, and this may compromise reproductive output. This could help explain why older females in the laboratory, but not the field, have higher abortion rates and produce smaller offspring (Lord et al., 2021). Importantly, when considering whether a pregnant female carries a larva to term, the timing of bloodmeals is key: in the field, females can choose the optimal time to feed depending on the size of their larva, whereas laboratory females may be offered blood when at a suboptimal stage of gestation. Lack of flexibility of feeding may thus explain higher abortion rates in the laboratory (Mellanby, 1937). The microbial community—which plays an important role in tsetse reproduction (Nogge, 1976; Alam et al., 2011)—of field versus laboratory flies is also different, which makes it hard to compare them directly. In general, findings in laboratory studies such as this give insights into the physiological processes and capacity of females, but parallel work on field-caught flies—where possible (see below for limitations)—would complement this work and validate the ecological relevance.

At the same time, it is important to appreciate the unique insights to be gained from such detailed laboratory studies, which would not be possible in a field setting. For example, the lack of data on egg abortions in tsetse has largely been due to the difficulty of observing reproductive output at this stage—which requires inspecting all tsetse-related debris from a breeding cage under a microscope. Such observations would not be possible in the field. Similarly, we were interested in investigating the consequences of abortion for subsequent reproduction, which requires longitudinally tracking the reproductive outputs of individual females. Such within-individual data are not

available in field studies, which limits inference as we cannot tease apart between-individual effects (e.g., better condition flies living longer) versus within-individual effects (e.g., previous birth outcome affects subsequent reproduction). When we considered such individual-level data, we find that—for females fed control (undiluted) blood—after having an abortion, they take longer to produce their next offspring, and these offspring are smaller. This suggests that abortion may be more likely due to physiological constraint than an adaptive strategy to improve subsequent reproductive output. This suggestion is tentative, however, given the weak effect and small sample size, and future work on this topic will be enlightening. Moreover, we do not find that the sex of viable offspring is affected by the age, nutritional state or reproductive history of the mother. We had predicted that females might strategically abort male offspring when they lack sufficient resources to produce a balanced sex ratio; but we note that this is built on our assumption that female tsetse are the sex with higher fitness returns. There is a need for more research to test this assumption and to develop theoretical predictions on whether sex biases may occur in tsetse depending on mode of sex determination, energetic requirements of males or females as well as their likely reproductive success.

Our study focuses on abortions (termed also prenatal mortality, reproductive failures or foetal/larval loss) in a viviparous fly. We provide new evidence showing that, when considering the developmental stage of offspring aborted, younger females are more prone to abort eggs and abortions increase overall with female age, as reported previously. We do not find strong evidence, however, that abortion is an adaptive strategy to improve subsequent reproductive output. As found in field studies, abortion rates are indicative of female physiological stress—here measured as nutritional deprivation. We appreciate the challenges of measuring such prenatal mortality as a reproductive outcome in the field (both in tsetse and other viviparous taxa). Such challenges not withstanding, we argue that this represents a key reproductive trait with important implications both for understanding age-dependent maternal allocation as well as how ecological adversity, and climate change, will impact population dynamics in these species.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GitHub, https://github.com/sineadenglish/tsetse-repro-loss.

## Author contributions

Project conception: SE, with input from AMGB, LRH and JSL. Data collection: LRH, RL and JSL. Data analysis and interpretation: SE, with input from all co-authors. SE led the writing of the manuscript, and AMGB, LRH, JSL, GAV, and JWH contributed. LRH created Figure 1. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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EDITED BY
Ann Valerie Hedrick,
University of California, Davis, United States

REVIEWED BY
Adam Hayward,
Moredun Research Institute,
United Kingdom
Stefano Mattioli,
University of Siena, Italy
Aaron Foley,
Texas A&M University Kingsville,
United States

*CORRESPONDENCE
Solène Cambreling
solene.cambreling@univ-lyon1.fr

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# Natal environmental conditions modulate senescence of antler length in roe deer

Solène Cambreling[1]*, Jean-Michel Gaillard[1], Maryline Pellerin[2], Cécile Vanpé[3], François Débias[1], Daniel Delorme[2], Rébecca Garcia[1], A. J. Mark Hewison[4] and Jean-François Lemaître[1]

[1]Laboratoire de Biométrie et Biologie Évolutive Unité Mixte de Recherche (UMR) 5558, Centre National de la Recherche Scientifique (CNRS), Université Lyon 1, Villeurbanne, France, [2]Office Français de la Biodiversité, Direction de la Recherche et de l'Appui Scientifique, Service Conservation et Gestion Durable des Espèces Exploitées, Châteauvillain, France, [3]Office Français de la Biodiversité, Direction de la Recherche et de l'Appui Scientifique, Service Conservation et Gestion des Espèces à Enjeux, Impasse de la Chapelle, Villeneuve-de-Rivière, France, [4]Comportement et Ecologie de la Faune Sauvage (CEFS), Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAe), Université de Toulouse, Castanet-Tolosan, France

It is now broadly admitted that female reproductive senescence – a decline in reproductive performance with increasing age – occurs in most species, at least among birds and mammals. Although information is more limited, male reproductive senescence has been regularly inferred from the decline in the size or performance of phenotypic traits that underly male reproductive success, particularly secondary sexual traits. However, the degree to which environmental conditions influence the pattern of senescence in sexual traits remains largely unknown. From the analysis of two long-term studies of populations of European roe deer (*Capreolus capreolus*) subjected to markedly different environmental contexts in the wild, we tested the hypothesis that harsh natal and/or current conditions should lead to earlier and/or stronger rates of senescence in the length of fully-grown antlers than good natal and/or current conditions. We found evidence of similar patterns of antler length senescence in both populations, with an onset of senescence around 7 years of age and a decrease of length by about 1–1.5 cm per additional year of life from 7 years of age onwards. We found that good early-life conditions delay senescence in antler length in roe deer. Our results also revealed that senescent males seem to be unable to allocate substantially to antler growth, confirming that antler size is, therefore, an honest signal of male individual quality. By modulating age-specific allocation to secondary sexual traits, natal and current conditions could influence female mate choice and male–male competition over mates, and as a result age-specific reproductive success, and should be accounted for when studying the dynamics of sexual selection.

KEYWORDS

***Capreolus capreolus*, reproductive ageing, secondary sexual traits, sexual selection, weapon**

## Introduction

From an evolutionary viewpoint, the process of reproductive senescence (also called "reproductive ageing") corresponds to a decline in reproductive performance with increasing age (Monaghan et al., 2008; Nussey et al., 2013; Gaillard and Lemaître, 2020). Given the wealth of recently accumulated empirical evidence, the widespread occurrence of female reproductive senescence in the wild is now unequivocal, at least for avian and mammalian species (Nussey et al., 2013; Lemaître et al., 2020a; Vágási et al., 2021). While various reproductive metrics have been used to evaluate female reproductive senescence, the number of viable daughters at birth produced by a female of age x (the "$m_x$" value) has been the most frequently studied and has been shown to decrease with increasing age in most mammals (Lemaître et al., 2020a). However, several components of this quantity have also been shown to decline over the lifetime (Lemaître and Gaillard, 2017), including birth rate (e.g. red squirrels, *Tamiasciurus hudsonicus*, McAdam et al., 2007), pregnancy rate (e.g. moose, *Alces alces*, Ericsson et al., 2001), and litter/clutch size (e.g. barn swallows, *Hirundo rustica*, Balbontín et al., 2012).

Currently, the information on male reproductive performance throughout the life course is far more limited due to the difficulties of having large samples of known-age animals (e.g. in mammalian species with typical female-biased adult sex ratio) and of assigning paternities in the wild (Lemaître and Gaillard, 2017). In polygynous and promiscuous species, male reproductive success can only be reliably assessed using pedigree information, especially when multiple mating and paternity occur, and the lack of required genetic data for determining paternity likely explains the low number of studies on age-specific reproductive success in males (Lemaître and Gaillard, 2017). Despite this, a few recent studies of paternity in vertebrates indicate that male reproductive senescence is likely pervasive in the wild (e.g. Widdig et al., 2004; Dugdale et al., 2011 for case studies).

Male reproductive success depends on the allocation of energy to pre-copulatory traits that confer an advantage in terms of male–male competition and/or female mate choice (i.e. secondary sexual traits such as colouration in peacock, *Pavo cristatus*, Dakin and Montgomerie, 2013; antlers in white-tailed deer, Morina et al., 2018) and post-copulatory traits that confer an advantage in male–male competition to fertilise females (e.g. testes size and sperm concentration, Schulte-Hostedde and Millar, 2004; sperm quality, Gage et al., 2004). In polygynous species, where males compete intensely for access to females, allocation to pre-copulatory traits is a key determinant of reproductive success as they may signal male genetic quality and/or confer an advantage during physical contests among males (Clutton-Brock, 1982; Berglund et al, 1996). So far, studies that focused on the occurrence of male reproductive senescence in terms of a decline in the size and/or performance of secondary sexual traits with increasing age have provided contrasting results (see Lemaître and Gaillard, 2017 for a review). For instance, although senescence has been reported in tail length in barn swallow (Møller and De Lope, 1999); number of antler points in red deer, *Cervus elaphus* (Mysterud et al. 2005); antler size in moose, *Alces alces* (Stewart et al. 2000); horn growth in ibex, *Capra ibex* (Von Hardenberg et al., 2004) and pigmentation in kestrels, *Falco tinnunculus* (Lopez-Idiaquez et al., 2016), many other studies have failed to detect senescence in male reproductive traits (e.g. song parameters in reed warbler, *Acrocephalus arundinaceus*, Forstmeier et al., 2006; antler mass in red deer, *Cervus elaphus*, Kruuk et al., 2002). Moreover, a given secondary sexual trait may senesce in certain species, but not in other closely related species (Lemaître and Gaillard, 2017; see Table 1).

Empirical evidence also suggests that environmental conditions modulate patterns of senescence among populations from the same species. So far, these studies have largely focused on actuarial senescence (i.e. an increase of mortality rate with age) (Holand et al., 2016; Cayuela et al., 2021) or on female reproductive senescence (e.g. Svalbard reindeer, *Rangifer tarandus platyrhynchus* in Douhard et al., 2016; Asian elephant, *Elephas maximus* in Mumby et al., 2015). A meta-analysis based on 14 bird and mammal species reported that the rate of reproductive senescence in females is, on average, lower when the conditions experienced during post-natal development are favourable (Cooper and Kruuk, 2018). This relationship was not statistically significant in males, possibly because of a lack of statistical power (effects size only available for four species in this meta-analysis). Yet, evidence that both current and early-life environmental conditions influence male reproductive traits during adulthood is widespread in the literature (e.g. stalk-eyed fly, *Cyrtodiopsis dalmanni* in Cotton et al., 2004; red grouse, *Lagopus lagopus scoticus* in Vergara et al., 2012) and studies evaluating how environmental conditions modulate the age-related decline in secondary sexual traits, both in terms of the strength (rate) and timing (age at onset) of male reproductive senescence are now required.

To fill this knowledge gap, we investigated among- and between-population differences in senescence of antler length in two populations of European roe deer (*Capreolus capreolus*) that experience markedly different environmental contexts. The antlers of cervids are one of the most conspicuous secondary sexual traits in the living world (Gould, 1974; Clutton-Brock, 1982; Lemaître et al., 2014a) even if the relative antler length substantially differs among Cervids, which is largely explained by the species-specific intensity of sexual selection (Geist, 1987; Geist, 1998). Antlers are used in aggressive contests between males (Clutton-Brock, 1982; Hoem et al., 2007) and as an honest signal of phenotypic quality (see e.g. Vanpé et al., 2007; Morina et al., 2018). In the highly polygynous red deer (*Cervus elaphus*), antler size is correlated with testes size, as well as both ejaculate quantity and quality, confirming the hypothesis that antler size is a reliable indicator of male fertility (Malo et al. 2005). In this study, we used antler length as a measure of antler size, as antler mass or volume cannot been measured on living individuals. Moreover, antler length is known to be associated with mating success in males in red deer (e.g. Kruuk et al., 2002) and roe deer (Vanpé et al., 2010) and currently constitutes a commonly used measure in comparative studies (Clutton-Brock, 1982; Plard et al., 2011). Antlers are deciduous sexual traits that require a huge allocation of energy to grow each year (Goss, 1970; Baxter et al., 1999), which is likely to impact short-term survival (Lemaître et al., 2018). Senescence in antler traits, especially length, has already been documented in many deer species *in natura* (see Table 1), including

TABLE 1 Studies reporting age-related antler metrics in cervids, in wild populations.

| Species | Population | Article | Studied trait | Senescence observed | Antlers state | Onset of senescence (in years) | Data type | Age | Age range (in years) | Longevity |
|---|---|---|---|---|---|---|---|---|---|---|
| Moose (*Alces alces*) | 6 regions in Alaska | Bowyer et al. (2001) * | Antler size | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | | | Antler spread | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | | | Palm width | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | | | Palm length | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | | | Number of tines | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | | | Beam circumference | Yes | NA | 12 | T | Cont. | 1–14+ | 16 (a) |
| | Northern Norway | Markussen et al. (2019) * | Antler size | No | Hard | – | L & T | Cont. | 2–11 | 16 (a) |
| | 3 regions in Norway | Saether and Haagenrud (1985) * | Number of antler points | Yes | Hard | [7.5–9.5] | T | Class | 1–13 | 16 (a) |
| | Alaska | Stewart et al. (2000) | Antler length | Yes | NA | 10 | T | Cont. | 1–16 | 16 (a) |
| | Yukon | Gauthier and Larsen (1985) * | Antler length | Yes | NA | 13+ | T | Class | 1–13+ | 16 (a) |
| | | | Antler spread | No | NA | – | T | Class | 1–13+ | 16 (a) |
| | | | Shaft circumference | No | NA | – | T | Class | 1–13+ | 16 (a) |
| | | | Palm width | No | NA | – | T | Class | 1–13+ | 16 (a) |
| Hainan Eld's deer (*Cervus eldi hainanus*) | Baisha | Nie et al. (2011) | Antler velvet mass | No | Velvet | – | L | Cont. | 2–11 | 11 |
| Red deer (*Cervus elaphus*) | Isle of Rum | Kruuk et al. (2002) | Antler mass | No | Hard | – | L | Cont. | 5–16 | 14 (a) |
| | | Nussey et al. (2009) | Antler length | No | Hard | – | L | Cont. | 3–14+ | 14 (a) |
| | | | Antler coronet circumference | Yes | Hard | 9 | L | Cont. | 3–14+ | 14 (a) |
| | | | Number of antler points | Yes | Hard | 11 | L | Cont. | 3–14+ | 14 (a) |
| | | Lemaître et al. (2014b) | Number of antler points | No | Hard | – | L | Cont. | 10–14+ | 14 (a) |
| | Bologna & Pistoia | Mattioli et al. (2021) * | Antler mass | Yes | Hard | 11 | T | Cont. | 1–14 | 14 (a) |
| | Norway | Mysterud et al. (2005) * | Number of antler points | Yes | Hard | 12 | T | Cont. | 2–16 | 14 (a) |
| | Warnham | Huxley (1931) * | Antler weight | No | Hard | – | T | Cont. | 1–14 | 14 (a) |
| | | | Number of points | Yes | Hard | 9 | T | Cont. | 1–14 | 14 (a) |
| | Western Carpathian | Smolko et al. (2022) * | Number of points | Yes | NA | 12 | T | Cont. | 2–13 | 14 (a) |
| | | | Beam length | Yes | NA | 13 | T | Cont. | 2–13 | 14 (a) |
| | | | Antler mass | Yes | NA | 13 | T | Cont. | 2–13 | 14 (a) |

*(Continued)*

TABLE 1 Continued

| Species | Population | Article | Studied trait | Senescence observed | Antlers state | Onset of senescence (in years) | Data type | Age | Age range (in years) | Longevity |
|---|---|---|---|---|---|---|---|---|---|---|
| Roe deer (*Capreolus capreolus*) | Chizé, Trois-Fontaines & Bogesund | Vanpé et al. (2007) | Antler size | Yes | Velvet | [8–12] | L | Class | 1–12 | 13 (a) |
| Black-Tailed Deer (*Odocoileus hemionus columbianus*) | Hopland | Thalmann et al. (2015) * | Antler size | No | NA | – | T | Cont. | 1–13 | 15 (c) |
| Sika deer (*Cervus nippon*) | Killarney | Hayden et al. (1994) * | Number of points | Yes | NA | 11 | T | Cont. | 1–12 | 19 (a) |
| | | | Beam length | No | NA | – | T | Cont. | 1–12 | 19 (a) |
| Wapiti (*Cervus canadensis nelsoni*) | ALE Reserve | McCorquodale et al. (1989) * | Beam girth | No | Hard | – | T | Cont. | 2–9 | 12 (a) |
| | | | Antler weight | No | Hard | – | T | Cont. | 2–9 | 12 (a) |
| | | | Antler points | No | Hard | – | T | Cont. | 2–9 | 12 (a) |
| European fallow deer (*Dama dama*) | Phoenix Park | Smith et al. (2021) | Antler width | No | Hard | – | L | Cont. | 1–11 | 12 (d) |
| | | | Antler length | Yes | Hard | 7 | L | Cont. | 1–11 | 12 (d) |

These studies were found using keywords [("age" OR "senescence") AND ("male*") AND ("antler*" or "weapon")] in Google Scholar and Web of Science. Some articles were also selected among those citing Vanpé et al. (2007) or Nussey et al. (2009). Some papers on the relationship between antler size and age have been withdrawn from the selection because the individuals studied were too young to allow detecting reproductive senescence, or because the individuals were supplementary fed. Data types are categorised as longitudinal (L) vs. transversal (T). Age is categorised as continuous (Cont) or in class. When different data from several populations were available, we either chose the same population as the one in the concerned article (if available) or the population with the biggest sample size. (a) see Lemaître et al. (2020b), appendix 3. (c) see Taber and Dasmann (1957). (d) see McElligott et al. (2002) *refers to animals hunted in their environment. NA, not available.

roe deer (Vanpé et al., 2007). However, Vanpé et al. (2007) analysed age-dependence in antler size using age classes, precluding an accurate assessment of the onset and rate of reproductive senescence.

Based on the above previous findings, we first tested the hypothesis that hard (i.e. fully-grown) antler length decreases with increasing age in adult roe deer males. In addition, we expected that current environmental conditions should affect the pattern of senescence of antler length, with males living in a harsh environment showing faster/steeper senescence in antler size than those living in a favourable environment. Furthermore, since better environmental conditions in early life seem to be associated with slower rates of reproductive senescence, at least in females (Cooper and Kruuk, 2018), we expected males born in good cohorts, that experience a high-quality natal environment, should exhibit later and/or less steep senescence in antler length than males born in poor cohorts.

# Material and methods

## Study sites, population monitoring and data collection

The European roe deer is a weakly polygynous cervid species characterised by a slight size dimorphism, with males being ca.10% larger than females (e.g. Vanpé et al., 2008). During winter, males are non-territorial, whereas they defend exclusive territories from spring to late summer (e.g. Johansson, 1996). Males carry deciduous antlers that grow back each year starting from the 1st year of life. Antlers of yearlings and adults are cast after the summer rut, in October or November, and immediately grow back until they are cleaned of velvet around February–March, corresponding to their final hard state. In their first year of life, males can grow short antler (called "buttons") atop their pedicles, before growing full antlers in following years (Sempéré, 1990). The photoperiod affects testosterone and luteinizing hormone (LH) concentration levels through a complex interaction, which in turn governs antler growth and maintenance, modulating the antler growth cycle (Sempéré et al., 1992).

We used data collected on roe deer from two enclosed forests located in France: Trois-Fontaines (Haute-Marne; 48°43′N, 4°55′E; 1,360 ha) and Chizé (Deux-Sèvres; 46°50′N, 0°25′W; 2,614 ha), both managed by the Office Français de la Biodiversité (OFB). These forests differ in terms of environmental conditions: rich soils and continental climate at Trois-Fontaines (i.e. highly productive environment) contrasting with the poor soils and oceanic climate at Chizé (i.e. weakly productive environment, Pettorelli et al., 2006). Roe deer at Trois-Fontaines and Chizé are intensively monitored by capture-mark-recapture (CMR) since 1975 and 1977, respectively, and for this study we used data from 1980 onwards at Trois-Fontaines and 1981 onwards at Chizé (because of the requirement for measurements of hard antlers on known-aged roe deer from 3 years of age onwards, see below). Winter capture sessions (from December until March) are organised each year by the OFB with the

help of many volunteers, lasting for about 10 days spread over 7 or 8 weeks. During a capture session, about 2.5 km of nets are set up to encircle a few forest plots. Roe deer are then captured in these nets and transported to the handling site located in the centre of the forest. At each capture, sex, body mass (± 50 g) and antler length (± 0.5 cm) of males are recorded. Antler length is measured from the lower edge of the coronet to the tip of the longest antler beam. Each roe deer of known age (i.e. marked either in their birth spring at a few days of age or during winter capture when they still have a milk premolar tooth) is marked with ear tags and a collar (color/letter coded, VHF and/or GPS) before being released on site (Gaillard et al., 1993). More than two thirds of individuals are of known age in each of the two populations. Due to management restrictions in the two study areas, known-age individuals are rarely removed from the populations, but are right-censored when this occurs. Harvest is not selective in neither Chizé nor Trois-Fontaines, and is allowed only for population control.

Captures take place in winter, during the antler growth period, so that some males are in hard antler, whereas others are still in velvet when caught. A preliminary analysis of our data based on animals that were frequently recaptured during a given season suggested that antlers may remain in velvet for several weeks once they have attained their final size. Hence, because of the existence of individual variation in antler phenology (see also Clements et al., 2010), we only analysed measurements of hard (i.e. fully-grown) antlers to circumvent the need to control for antler growth. Also, preliminary analyses indicate that old males cast their antler later than young ones, which is beyond the scope of this study although it would be of particular interest for future investigations (see Figure S1, Tables S8, S9). There is, however, no reason to expect that the later shedding of velvet by older males would affect the outcomes of our analyses. We used the total sum length of the right and left antlers for a given individual as a measure of antler size. As broken antlers often result from human-related factors, they do not reflect any biological feature of the individuals. We thus attributed the measurement of intact antlers to broken or missing antlers (see also Lemaître et al., 2018). This implementation was justified by the very strong correlation between left and right antler lengths among males (Pearson coefficient of correlation, r = 0.88 [0.85;0.90], N = 325). Finally, since we focused on senescence, we only used antler measurements of males that were at least 3 years of age during the rut (captured during their third year), which corresponds to the age when most males sire fawns for the first time (Vanpé et al., 2009) and when most males have almost reached their full antler size (Vanpé et al., 2007).

## Statistical analyses

We performed our analyses on both absolute and relative (to body mass) antler length. Indeed, as antler size is subject to strong positive allometry (Plard et al., 2011) like most ornaments (Kodric-Brown et al., 2006), we replicated all the analyses using antler length relative to body mass. We did not standardise adult male body mass for a certain date since we did not detect any change of mass with Julian date (slope of the linear regression between body mass and Julian date ± SE = −0.010 ± 0.15, t = 32.53, P = 0.486 and 5.884 e-4 ± 9.295 e-3, t = 0.063, P = 0.95 for Trois-Fontaines and Chizé, respectively, Figure S2), in line with the income breeder life history tactic displayed by roe deer (Andersen et al., 2000). For relative antler length, we used the same analytical procedure as described below, except that antler size was log-scaled and body mass was systematically included (on a log scale also) as a covariate in all models. For both absolute and relative antler length, we selected the best model describing age-specific changes in antler length through a comparison of linear mixed effect models, with antler length as the dependent variable, age as the covariate, and the individual identity and year as random effects on the intercept to account for repeated measures of a given individual and annual variations, respectively. We used the R package *lme4* (Bates et al., 2015). We compared models that included no age effect (constant antler size), a linear effect of age, a quadratic effect of age, a full-age model (one parameter for each year of age) and a previously selected threshold age model (see below). To control for the confounding effect of the possible selective disappearance of males with small antlers, which could mask senescence at the population level, we sought to incorporate individual longevity as covariate in our models for hard-antlered males. Unfortunately, this information was not available for most males in our dataset and we thus used the age at last capture (corresponding to the age at which we last controlled a given individual) instead, as recommended by Nussey et al. (2008) (see also Van de Pol and Verhulst, 2006).

The threshold model included in the model selection procedure described above was previously identified by estimating the intercept (age of onset of senescence) and slope with their confidence intervals. Basically, we fitted threshold age models using three possible forms: (1) a constant antler length up to a threshold age after which antler length decreases with increasing age; (2) an increase in antler length up to a threshold age after which antler length remains constant; and (3) an increase in antler length up to a threshold age after which antler length decreases with increasing age. When the best model included a threshold age beyond which we did not have any data, we selected the second-best threshold model. Once the best threshold age was estimated, we used the R package *OnAge* (Jacob et al., 2017) to estimate the confidence interval on the onset of antler senescence.

To investigate the effect of current environmental conditions on antler length senescence, we first identified a population-specific senescence model and then estimated the onset and the rate of senescence (because the two populations face different ecological conditions, see above). Because the onset of senescence did not occur at the same age in both populations in the retained models (see Results), we performed a z test to compare regression coefficients and evaluate differences in the rate of senescence (see Paternoster et al., 1998). Given the limited support for senescence at one site, we also pooled data from the two populations to compare senescence rates (for both absolute and relative antler length) between populations using additive and interactive effects of population and age.

To test for the influence of natal conditions on antler length senescence, each individual was assigned a cohort quality value corresponding to the mean winter body mass of fawns (at 8 months

of age) born in that individual birth year, corrected for sex (males taken as reference) and date of capture (standardised on 30$^{th}$ January). Fawn body mass (at 8 months of age) is impacted by climatic conditions and population density (Toïgo et al., 2006), thus serves as an integrative and suitable measure to assess the quality of the year and the influence of these variables on juvenile roe deer. Additionally, this metric is particularly reliable because of the overwhelming influence of cohort variation over current conditions on adult body mass in roe deer (Pettorelli et al., 2002).

In the following analyses, the quality of a given cohort was determined as being either low (i.e. mean fawn winter mass lower than the median among cohorts) or high (i.e. higher than the median among cohorts), with the median being determined separately for each population. Following the same logic as previously described, we evaluated the pattern of reproductive senescence for each cohort quality (i.e. low vs. high) to compare the onset and rate of reproductive senescence in relation to environmental conditions in early life.

Model selection was consistently based on the Akaike Information Criterion (AIC), using the correction for sample size (AICc) to avoid data overfitting (Burnham and Anderson, 2004). All analyses were performed using the R software (version 4.1.2; R Development Core Team, 2015) and all results are reported as parameter estimate ± SE. Coefficients of determination for the models were reported as conditional (providing the proportion of variance explained by both the fixed and random factors). All data and script for the analyses are available in supplementary information.

# Results

## Senescence of antler length

Our dataset contained fully-grown antler length measurements for 213 males (223 measurements of 142 males at Chizé and 111 measurements of 71 males at Trois-Fontaines). Antlers were on average 11.5% longer at Trois-Fontaines than at Chizé (sum of both antlers: mean of 417 ± 4.76 mm vs. 369 ± 3.43 mm; $t_{224} = -8.208$, $P < 0.001$). There were few data on very old individuals for either population in our dataset: for males 10 years of age or older, we obtained 11 measurements for 9 individuals at Chizé and 4 measurements for 4 individuals at Trois-Fontaines.

In Chizé, the selected model of age-specific changes in antler length was the threshold model (Tables S1–S6, Figures 1A, B). Absolute antler length started to decrease by about 1.4 cm each year from 7.28 [5.37; 8.97] years of age onwards (slope ± SE = −14.39 ± 4.02 mm, $P < 0.001$, $R^2 = 0.50$) or by 1.35 cm when the allometric effect of body mass on antler length was accounted for (i.e. relative antler length), from 7.22 [5.12; 8.84] years of age onwards (slope ± SE = −0.04 ± 0.01 on a log scale, $P < 0.001$, $R^2 = 0.46$).

In Trois-Fontaines, the baseline model (i.e. only including the age at last capture) was retained: no detectable diminution of antler length with increasing age was detected at first sight using either absolute or relative antler length (Tables S2–S6, Figures 1C, D). However, a threshold model allowing increased antler length throughout the prime adulthood provided the best fit (AICc = 1178.76 and −152.89 vs. 1179.09 and −152 for the constant model for absolute and relative antler length, respectively). The absolute antler length increased by approximately 0.7 cm each year from 3 to 6.84 [3; 11] years of age (slope before the threshold age ± SE = 7.23 ± 3.68 mm, $P = 0.05$), and then decreased by about 1 cm per additional year of age (slope beyond the threshold age = −10.67 ± 5.97, $P = 0.08$). When accounting for the allometric effect of body mass, antler length increased until 6.67 [3; 11] years of age, and then decreased by approximately 0.9 cm per additional year of age (slope before the threshold age ± SE = 0.02 ± 0.01 on a log scale $P = 0.03$; slope beyond the threshold age = −0.02 ± 0.01 $P = 0.11$, $R^2 = 0.71$). Although the effect sizes of these models were not statistically different from 0, these models provided a satisfactory fit of the data ($R^2 = 0.63$ and 0.71 for absolute and relative antler length, respectively), with a very similar explanatory power to what we reported at Chizé. The rate of decline of antler length tended to be lower in Trois-Fontaines than in Chizé although no significant differences were detected in neither absolute (1.07 ± 0.6 vs 1.44 ± 0.4 cm lower per year of age, $z_{210} = -0.51$; $P = 0.60$) nor relative antler length analysis (−0.02 ± 0.01 vs −0.04 ± 0.01, $z_{294} = -1.41$; $P = 0.16$).

We did not find any statistical support for a difference in the age-dependent pattern of antler length between populations (i.e. no evidence of any interactive effect of age and population, Table S3). In both populations, antler length declined linearly from around 7 years of age onwards. We therefore conducted the following analyses by pooling the two populations and testing the additive effect of population in our model selection.

## Natal environment and senescence of antler length

On average, fully-grown antler length of males from 3 years of age onwards did not differ between low- and high-quality cohorts (386 ± 4.07 mm vs. 382 ± 4.30 mm in high- and low-quality cohorts, respectively; $t_{291} = 0.69$; $P = 0.49$).

In high-quality cohorts (N = 117 measures on 79 individuals), a decrease in absolute antler length with increasing age was observed from 9.33 [5.23; 13.00] years of age, with a decrease of about 2.5 cm per additional year of age (slope ± SE = −2.5 ± 1.0, $P = 0.02$, $R^2 = 0.59$, with an additive effect of population). The same pattern was observed once the allometric effect of body mass was included, with a diminution of antler length from 9.60 [5.70; 13.00] years of age and a decrease of 2.7 cm per additional year of age (slope ± SE = 0.08 ± 0.03, $P = 0.01$, $R^2 = 0.52$; see Figure 2, Tables S5–S7).

In low-quality cohorts, however, the age threshold model was not selected for either absolute or relative antler length analyses (full-age model selected with additive effect of population, see Figure 2, Tables S4–S7). The age threshold model was close to the full age model (ΔAICc = 2.5) for absolute antler length, and showed the existence of a strong senescence for both absolute and relative antler length with a slope (± SE) of −12.2 (± 4.49, $P = 0.007$, $R^2 = 0.62$) and of −0.03 (± 0.01, $P = 0.01$, $R^2 = 0.57$), respectively. Antler length decreased from about 7 years of age, starting earlier in low- than high-quality cohorts. Slopes of both absolute and relative antler length in high-quality cohorts were strongly influenced by a single measurement (see Figures 2C, D).

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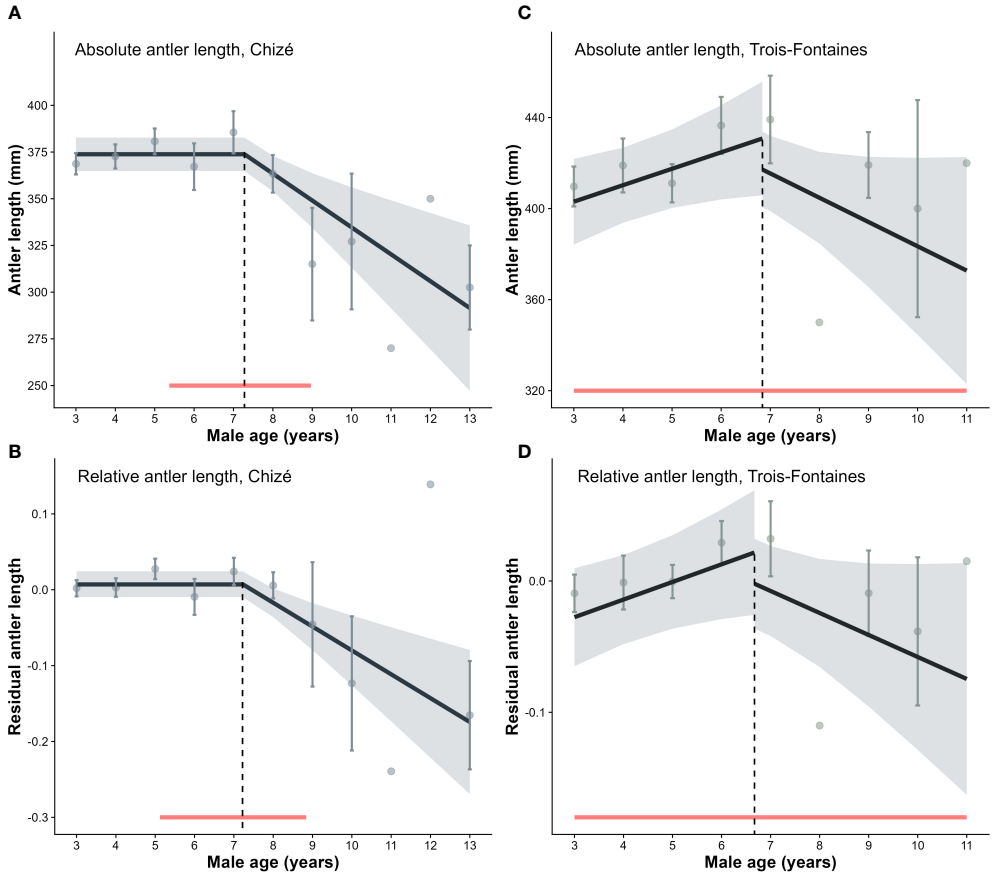


FIGURE 1
Patterns of age-specific changes in antlers in Chizé and Trois-Fontaines. **(A)** Absolute antler length in relation to age: age threshold set at 7.28 years old in Chizé (N = 223 measurements of 142 individuals). **(B)** Relative (to body mass) antler length in relation to age: age threshold set at 7.22 in Chizé. **(C)** Absolute antler length in relation to age: age threshold set at 6.84 in Trois-Fontaines (N = 110 measurements of 71 individuals). **(D)** Relative (to body mass) antler length in relation to age: age threshold set at 6.67 in Trois-Fontaines. Red bands correspond to the confidence intervals for the age at the onset of senescence. Light bands correspond to 95% confidence intervals. Residual antler length was obtained by extracting residuals from a regression model of antler length vs. body mass (both log-transformed).

# Discussion

## Senescence in antler length in populations facing different environmental contexts

Our study highlights the occurrence of senescence in the length of fully-grown antlers in roe deer, as evidenced by the decrease in both absolute and relative antler length with increasing age at Chizé and Trois Fontaines, starting at about 7 years of age (see Figure 1). Interestingly, while antler size was constant during the prime adulthood at Chizé, it increased with age until the onset of senescence at Trois-Fontaines. However, it is important to note that our dataset contains a low number of individuals older than 7 years of age, which may limit our ability to accurately quantify senescence patterns. Further data will be required to confirm this pattern. Our findings support Vanpé et al.'s (2007) study that reported evidence for age-specific variation in the length of velvet antler at a given date in both study populations, with a decrease between prime-aged (2–7 years old) and senescent males (8+ years old).

Antler senescence has previously been reported when studying fully grown antlers in more dimorphic and polygynous cervid species *in natura*, such as moose (Saether and Haagenrud, 1985), fallow deer (*Dama dama*, Smith et al., 2021) and red deer (Mysterud et al., 2005) (see also Table 1). In managed populations, "antler/trophy regression" (see e.g. Gauthier and Larsen, 1985) or "Zurücksetzen" (see e.g. Drechsler, 1980) or "ravalement" (Agence nationale des chasseurs de grand gibier, 2005) also has frequently been mentioned in roe deer, fallow deer and red deer, but it has never been firmly assessed by quantitative analyses based on detailed individual monitoring. Our results provide evidence that senescence in antler size might be widespread across deer species irrespective of the intensity of sexual selection. Thus, despite a modest sexual size dimorphism and a low level of polygyny, roe deer males cannot maintain the same allocation to antlers throughout their entire lifetime.

Our present analysis shows that the onset of antler length senescence takes place at about 7 years of age in roe deer, supporting Vanpé et al.'s study in which this onset was fixed *a priori* based on the onset of actuarial senescence. Our study thus indicates that senescence in antler size (this study) and actuarial

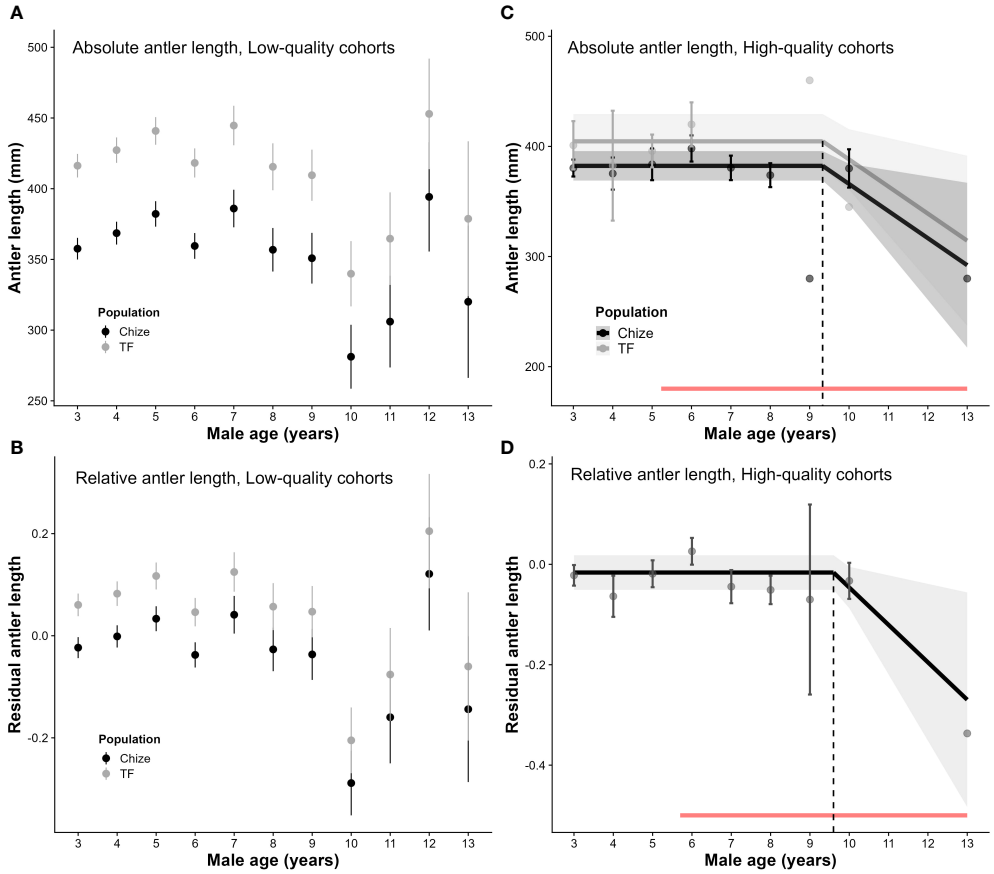


FIGURE 2
Patterns of age-specific changes in antlers in low- and high-quality cohorts. **(A)** Absolute total antler length with age in low-quality cohorts (i.e. with a mean yearling mass below the median over the study period): full-age model (N = 217 measures of 134 individuals). **(B)** Relative total antler length with age in low-quality cohorts: full-age model. **(C)** Absolute total antler length with age in high-quality cohorts (i.e. with a mean yearling mass above the median over the study period): threshold model with an onset of senescence at 9.33 years of age (N = 117 measures of 79 individuals). **(D)** Relative total antler length with age in high-quality cohorts: threshold model with an onset of senescence at 9.6 years of age. Red bands correspond to the confidence intervals for the age at the onset of senescence. Grey bands correspond to 95% confidence intervals. Residual antler length was obtained by extracting residuals from a regression model of antler length vs. body mass (both log-transformed).

senescence (Gaillard et al., 1993; Loison et al., 1999) are indeed synchronous in roe deer males, supporting the hypothesis that antler size is an honest signal of male phenotypic quality (Vanpé et al., 2007), at least when resources are limiting. In addition, given that male reproductive success declines with increasing age from 8 years onwards (Vanpé et al., 2009), our results suggest that senescence in antler size is one possible proximate mechanism driving impaired reproductive success among old males. Indeed, phenotypic traits such as the size of secondary characters might serve as an indicator of fertility ("phenotype-linked fertility hypothesis", Sheldon, 1994), as observed in red deer where males with the longest antlers produce a higher quantity of sperm, as well as more rapid sperm (Malo et al., 2005). It would now be particularly interesting to analyse patterns of senescence in male fertility measured by various ejaculate-related traits to test the hypothesis that patterns of senescence for pre- and post-copulatory traits are aligned.

Interestingly, the allocation to antler size was substantially larger (>10%) at a given age at Trois Fontaines compared to Chizé. This pattern is likely explained by differences in the environmental context between populations. Environments with abundant resources, such as at Trois-Fontaines, should allow roe deer males to grow longer antlers (this study), develop higher body mass (Hewison et al., 2011) and attain better physiological performance (e.g. immunocompetence, Cheynel et al., 2017) compared to environments with more limited resources such as Chizé. Our results thus suggest that males adjust the life course of their antler size to available resources. In particular, old males in favourable environments allocate more resources in absolute terms to the development and maintenance of their secondary sexual traits than they do in less suitable environments. Whether environmental conditions might explain the lack of senescence in antler size in some populations of red deer (Nussey et al., 2009), of sika deer (*Cervus nippon*, Hayden et al., 1994) or of black-tailed deer (*Odocoileus hemionus*, Thalmann et al., 2015) is yet to be investigated, despite the potential confounding effect of the management practices (e.g., hunting selection and pressure) in some populations. Importantly, patterns of senescence may be asynchronous among antler traits (see Table 1, e.g. Smith et al., 2021).

## Natal environmental conditions modulate senescence patterns in antler length

The impact of early-life conditions on male secondary sexual traits has previously been well documented, with good natal conditions generally improving the size of the trait during adulthood (e.g. in zebra finch, *Taeniopygia castanotis:* beak color and cheek patch size in Wilson et al., 2019; plumage ornaments in Naguib and Nemitz, 2007), but their impact on the senescence of secondary sexual traits had not yet been studied. Our results suggest that natal environmental conditions modulate patterns of senescence in antler length in both populations, although, notably, these senescence trajectories were mostly driven by the oldest males from Chizé. For poor-quality cohorts, a marked decrease in both absolute and relative (to body mass) antler length occurred from 7 years of age onwards (see Figure 2). That this pattern was not fully supported on statistical grounds comes from the low sample size of males older than 10 years of age, with only one individual at 12 and one at 13 years of age. In contrast, the decrease in both absolute and relative antler length with increasing age occurred somewhat later in high-quality cohorts, from 9.5 years onwards. Note, however, that this decrease was mostly driven by one old male with very small antlers at Chizé, leading the exact age of the onset of antler senescence of males born under favourable conditions to be difficult to determine. As in Trois-Fontaines, the very small sample size of animals older than 10 years of age prevented us to draw firm conclusions regarding the onset of antler length senescence in high-quality cohorts. According to a preliminary analysis (Tables S8, S9, Figure S1), the timing of velvet shedding seems to be delayed with age, while the opposite relationship has been documented in other cervids (e.g. Gómez et al., 2022 in red deer). In our study, this may partly explain the low number of old males (> 10 years of age) in our dataset. We cannot, therefore, conclude that senescence in antler length did actually occur for high-quality cohorts, although it appears that poor natal conditions lead to earlier age at onset of antler length senescence.

Natal conditions in roe deer strongly influence phenotypic traits such as body mass or jaw length (Hewison et al., 1996; Hewison et al., 2002; Pettorelli et al., 2002; Kjellander et al., 2006). The delayed senescence in antler length we report in males from high-quality cohorts suggests that favourable natal conditions also promote the expression of secondary sexual traits later in life, so that mature males are able to continue to allocate a lot to antler growth, even when old (i.e. > 7 years). Under the assumption that antler length reflects mating success (e.g. through the acquisition and maintenance of a high-quality territory) and fertilisation success (see above, Vanpé et al. 2010), the later onset of senescence in antler length for males born from high-quality cohorts might indicate a later onset of senescence in terms of reproductive success.

In contrast, males that experience limiting conditions in early life may suffer from earlier senescence in antler length as a result (see Figure 2), both in absolute and relative (to body mass) terms. The observed decrease in relative antler length with increasing age suggests that once senescence has started, males allocate less energy to antler growth for a given mass. As described above, this could either reflect the inability of old males to maintain their level of allocation to antler growth because of their poor physiological condition, or a tactic favouring the allocation of resources towards other traits in late life (e.g. gamete quality, somatic maintenance) (Maklakov and Immler, 2016). Finally, it would be valuable to study senescence in other antler characteristics (e.g. coronet circumference, casting date) which could potentially show a higher sensitivity to environmental conditions than antler length (Michel et al, 2016).

## Conclusions

Our study demonstrates that even in a weakly dimorphic species such as roe deer, antlers remain honest signals of individual quality and are sensitive to early-life environmental conditions. Based on our results, adverse natal conditions induce earlier and stronger antler length senescence in roe deer, which could potentially influence female choice and male–male competition, and as a result reduce mating success among old males. Antler length is an honest signal of individual quality in roe deer (Vanpé et al., 2007), and a possible indicator of fertilisation capacity in adults, because antler size correlates with testes size and sperm velocity, as shown by Wahlström (1994) in yearling roe deer and by Malo et al. (2005) in adult red deer. Therefore, we suggest that females should prefer mating with males with longer antlers (as in white-tailed deer: Morina et al., 2018), and should mostly avoid mating with old males to limit the fitness costs of reproductive senescence in their partner. The results of Vanpé et al.'s (2019) study on female mating tactics in roe deer are in line with this assumption: old females seem to avoid mating with old males, contradicting the long-held view that old males should be preferred by females (see Johnson and Gemmell, 2012 for a review).

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by Director of Food, Agriculture and Forest. The protocol of capture and blood sampling under the authority of the Office Français de la Biodiversité (OFB) was approved by the Director of Food, Agriculture and Forest (Prefectoral order 2009–14 from Paris). The land manager of both sites, the Office National des Forêts (ONF), permitted the study of the populations (Partnership Convention ONCFS-ONF dated 2005-12-23). All experiments were performed in accordance with guidelines and regulations of the Ethical Committee of Lyon 1 University (project DR2014-09, June 5, 2014).

## Author contributions

SC, J-MG, and J-FL designed the study with input from AJMH. SC, J-MG, CV, RG, FD, DD, MP and J-FL collected the data. SC analysed the data. SC wrote the first draft with inputs from J-MG, AJMH, and J-FL. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1139235/full#supplementary-material

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EDITED BY
Jean-Michel Gaillard,
Université Claude Bernard Lyon 1, France

REVIEWED BY
Roi Dor,
Open University of Israel, Israel
Sandra Bouwhuis,
Heligoland Bird Observatory, Germany
Karen Wiebe,
University of Saskatchewan, Canada

*CORRESPONDENCE
Héloïse Moullec
heloise.moullec@gmail.com
Pierre Bize
pierre.bize@vogelwarte.ch

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# Aging trajectories are trait- and sex-specific in the long-lived Alpine swift

Héloïse Moullec[1]*, Sophie Reichert[1] and Pierre Bize[2]*

[1]Department of Biology, University of Turku, Turku, Finland, [2]Anthropogenic Effects Research Group, Swiss Ornithological Institute, Sempach, Switzerland

Senescence is defined as the general deterioration of the organism (i.e. physiology, morphology, reproduction), and is associated with increasing mortality and decreasing fertility with age. Although senescence has now been widely reported in wild animals, little is known on whether senescence affects all traits, whether this process is synchronized across traits, and whether males and females are affected in the same way. Using an individual-based monitoring of 20+ years in free-living population of Alpine swifts (*Tachymarptis melba*), we investigated age-dependent variation between sexes and between six biometric traits, 4 reproductive traits, and 1 measure of parasite burden. We accounted for selective disappearance and terminal effects in our analyses. Our results provide general support for age-dependent variation at adulthood in 8 out of the 11 traits investigated. Most traits showed a variation with 2 thresholds, with first a strong improvement until 4 to 12 years of age (e.g., increased fork length, decreased parasite load, or earlier laying date) followed by a plateau and a decline at older ages. The age of the second threshold showed sex specific asynchrony, with an earlier threshold in males than in females for tail length, parasite burden and laying date, as well as moderate asynchrony across traits. Rates of senescence differed between sexes, with stronger senescence of the tail in females than in males and with evidence of reproductive senescence in females but not in males. We also found evidence of terminal investment in males with respect to brood size at hatching and terminal decline with increased asymmetry of the fork and decreased body mass. We found evidence of selective appearance with males with longer fork and little fork asymmetry starting to reproduce earlier in life, and females that start to reproduce earlier tending to higher reproductive success. Finally, we found selective disappearance of males with longer tails and marginal effect of selective disappearance of females with lower body mass. We discuss how natural or sexual selection may have led to these trait- and sex-specific patterns of aging in this long-lived bird.

KEYWORDS

**life-history theory, senescence, long-lived species, natural selection, selective disappearance, terminal effect, *Tachymarptis* (Apus) *melba***

## Introduction

Senescence is the age-related decline of phenotypic traits, that can be measured at the whole organism from a decline in morphological traits and physical performances, such as grip strength in humans (Sayer and Kirkwood, 2015) or number days spent rutting in red deer (*Cervus elaphus*; Nussey et al., 2009), to a decline in survival and reproduction (Bouwhuis et al., 2012; Nussey et al., 2013; Hayward et al., 2015; Bouwhuis and Vedder, 2017; Cooper et al., 2021). Although signs of senescence were once thought to be restricted to humans and laboratory models (Kirkwood, 2002; Masoro and Austad, 2005), senescence has now been widely reported in natural populations of animals (Nussey et al., 2008; Nussey et al., 2013; Fletcher and Selman, 2015). Studying senescence in the wild, however, is difficult because it requires long-term longitudinal data collected from large numbers of individuals of known age that live long enough to observe signs of senescence (Nussey et al., 2008). Despite an increasing number of longitudinal data on wild populations of vertebrates (Clutton-Brock and Sheldon, 2010), most studies on senescence to date are primarily limited to survival and reproductive traits (Nussey et al., 2013; Bouwhuis and Vedder, 2017; Lemaître and Gaillard, 2017; Lemaître et al., 2020). Many studies are also limited to female reproductive aging (Nussey et al., 2013), with male reproductive success often more difficult to measure due to uncertainties about paternity (Bouwhuis et al., 2009; Bouwhuis et al., 2010). This bias in single-trait and single-sex studies may limit our understanding of senescence for a given species as well as across species (Lemaître and Gaillard, 2017).

As opposed to the first hypotheses of evolutionary biologists, presuming that natural selection should synchronize senescence between different traits (Williams, 1957; Maynard Smith, 1962; Williams, 1999), studies in humans and laboratory animals have shown that senescence of different traits can often be uncoupled (Burger and Promislow, 2006; Martin et al., 2007; Nussey et al., 2009; Bansal et al., 2015; Moorad and Ravindran, 2022). A growing number of studies provide similar evidence of asynchronous senescence between traits in wild animals (Bouwhuis et al., 2012; Nussey et al., 2013; Hayward et al., 2015; Bouwhuis and Vedder, 2017; Cooper et al., 2021; Fay et al., 2021). However, much remains to be done to understand which traits are senescing first and why (Moorad and Ravindran, 2022). This research question is important to investigate whether, for example, reproductive senescence is induced by a decline in foraging ability, which in turn is induced by a decline in muscle mass and physical activity. This would predict an earlier onset of senescence in mass versus foraging ability versus reproduction.

Although there are fewer studies on male than female senescence in the wild, evidence of senescence was found in both sexes and, more interestingly, males are often found to senesce faster than females in mammals (Nussey et al., 2013). Several evolutionary hypotheses have been proposed to explain differences between the sexes in their rate of senescence. One of the best-known factors leading to sex-specific rate of senescence is a difference in their exposure to environmentally induced mortality, with the sex with greater mortality expected to senesce at the fastest rate (Williams, 1957; Bonduriansky et al., 2008). Accordingly, males in polygynous species often suffer higher mortality, as a result of male-male competition, and display faster rates of senescence (Lemaître et al., 2020; Bronikowski et al., 2022). Less is known however on sex-specific rates of aging in socially monogamous species (Nussey et al., 2013). As these species often show lower sexual size dimorphism and more balanced reproductive allocation between the sexes, sex-specific senescence is less expected in monogamous species than in polygynous ones (Promislow, 2003; Clutton-Brock and Isvaran, 2007). Studies in monogamous species are however showing a great diversity in the rates of senescence between the sexes, from no differences between the sexes in socially monogamous and cooperative breeding meerkats (*Suricata suricatta*; Thorley et al., 2020), to faster rates of senescence in male wandering albatrosses (*Diomedaexulans*; Pardo et al., 2013), male white-tailed eagles (*Haliaeetus albicilla*; Murgatroyd et al., 2018), and male red wolves (*Canis rufus*; Sparkman et al., 2017), and finally faster senescence in female common guillemots (*Uria aalge*; Reed et al., 2008). Sex-specific rates of senescence in monogamous species may be influenced by the life-history strategies adopted by each sex, and thus by the trade-offs involved in these strategies (Nussey et al., 2008; Lemaître and Gaillard, 2017). A greater allocation of one sex to parental care, or greater allocation in physiologically demanding activities (e.g. growth, foraging, territorial defense, secondary sexual characteristics) would thus increase its senescence rate (Reid et al., 2003; Reed et al., 2008; Nussey et al., 2009; Murgatroyd et al., 2018). To reach a better understanding of the variation of senescence in the wild, more studies investigating sex-specific senescence patterns are needed, especially in monogamous species, and ideally on phenotypic traits other than just reproduction (Nussey et al., 2009; Nussey et al., 2013).

Finally, when studying age-related variation among traits and sexes in free-living populations, it is also essential to account for heterogeneity between individuals in their rates of aging (Vaupel et al., 1979). In other words, it is essential to separate within- and between-individual effects on age-related changes (van de Pol and Verhulst, 2006). Indeed, age-related changes observed at the population level can be induced by within-individual changes (improvement early in reproductive life and senescence later in life) as well as by between-individual changes (selective appearance and disappearance of certain phenotypes; van de Pol and Verhulst, 2006). Between-individual effects, such as, for example, the early disappearance from the population of individuals with a low reproductive success (i.e. selective disappearance of poor breeders), were highlighted in most avian studies on senescence (Vedder and Bouwhuis, 2018) and can mask the decline of reproductive traits due to actual senescence (van de Pol and Verhulst, 2006; Nussey et al., 2008). Researchers thus need to control for demographic effects when aiming to measure actual senescence. Similarly, before the death of individuals, the age-related variation of fitness traits can change direction due to terminal investment (Clutton-Brock, 1984; Froy et al., 2013), with an expected increase of performance the last year of life, or due to terminal illness (Coulson and Fairweather, 2001; Rattiste, 2004), with an expected decrease of performance the last year of life. These terminal effects can also mask or be mistaken for a gradual age-related decline due to senescence,

especially in short-lived species. In recent years, authors investigating senescence in the wild increasingly account for between-individual variations by assessing selective disappearance, as well as terminal effects (Bouwhuis et al., 2009; Martin and Festa-Bianchet, 2011; Froy et al., 2013; Hayward et al., 2015; Zhang et al., 2015; Dingemanse et al., 2020; Cooper et al., 2021) and emphasize the need to control for these effects in studies on senescence.

In this study, we investigated sex and trait differences in aging trajectories in the socially monogamous and long-lived Alpine swift (*Tachymarptis melba*). Data were collected for 11 traits in both sexes throughout their reproductive lives. This longitudinal approach allowed testing for age-dependent variations while accounting for selective disappearance and terminal effects. Alpine swifts start breeding at 2 to 4 years of age (median age at first reproduction is 3 years of age; Tettamanti et al., 2012) and may live up to 26 years (median lifespan of breeders is 7 years of age; Arn, 1960; Bize et al., 2009). This migratory bird is socially monogamous and reproduces in colonies of up to several hundred pairs. Males and females provide equal parental care during the breeding period (Bize et al., 2004b), and previous studies found no difference in survival between the sexes (Bize et al., 2008a; Bize et al., 2014). Females lay a modal clutch of 3 eggs, and both sexes then incubate the eggs for 20 days and feed their nestlings up to fledging, which occurs 50 to 70 days after hatching (Bize et al., 2004b). Although males have a slightly larger body size than females (1% for wing length to 7.5% for fork length) (Bize et al., 2006a; Table A1 in appendix), this bird is sexually monomorphic to human eye, and molecular tools are required to reliably sex individuals.

The Alpine swift is an interesting model to explore differences in senescence between the sexes and asynchrony across traits for two reasons. First, as this bird species appears to be socially monogamous and sexually monomorphic, with both sexes equally involved in reproductive tasks, it can be expected that there will be few sex differences in senescence rates compared to polygynous species and species with high sexual size dimorphism, if differences in reproductive allocation are the main driver of sex-specific senescence rates. Second, as the Alpine swift is an extreme case of aerial bird species where adults spend most of their lives flying (they can fly up to 200 days without landing; Liechti et al., 2013), we can expect differences across traits in selection pressures and their rates of senescence (Moorad and Ravindran, 2022). This might be particularly true for traits, such as tail and wing length and symmetry, which have strong consequences on aerodynamics and energy expenditure during flight (Thomas, 1993a; Thomas, 1993b; Norberg, 1995a). Differences in the onset and rate of senescence between morphometric traits (body mass, wing length, tail length, tail asymmetry) and reproductive traits (laying date, clutch size, brood size at fledging) remain however rarely investigated.

# Material and methods

## Study population and general methods

The data were collected between 1999 and 2022 in two Alpine swift colonies located under the roof of two old buildings in Biel (Stadtkirche) and Solothurn (Bieltor), Switzerland. During this study period, the number of breeding pairs remained constant in Solothurn (about 50 pairs), whereas in Biel it declined from about 100 pairs to 60 pairs. Since 1999 there has been an intensive individual-based monitoring of adults and their reproductive success in both colonies. Fledglings are primarily recruited locally (i.e. 60% of the recruits), and recruits do not disperse after starting to breed in a colony (no "breeding dispersal"; Bize et al., 2017). Furthermore, as the annual capture probability of birds after they start breeding is virtually 1 (SE = 0.003) (Bize et al., 2006a), there is exceptional repeated information throughout the breeding life of adults. In this population, 68.8% of the breeders have been ringed as nestlings and are thus of known age. If a breeder is not captured for 2 consecutive years, it is considered dead (Bize et al., 2009).

Each year, nests were regularly visited to record laying dates, clutch sizes, and brood sizes at hatching and fledging, and to ring the nestlings. Adults were captured by hand while sitting on their eggs or brooding nestlings. As in the Alpine swift both parents must incubate the clutch and provision the brood, the brood size at hatching and fledging depends on both parents. Although we have little information in this species regarding the contribution of each sex to pre-hatching traits (i.e. laying date and clutch size), males may have thus an indirect impact on pre-hatching traits if females allocate differently in reproduction according to the quality (i.e. age) of their mate, as seen in other bird species (Komers and Dhindsa, 1989; Johnsen et al., 2005; Michl et al., 2005; Segami et al., 2021).

Adults were individually identified with a ring they received as nestlings or when they were first captured as adults and had not been ringed before. Each year when an adult was captured, we measured with a ruler to the nearest mm: wing length on the flattened and straightened closed wing, tail length from the base to the tip of the outermost tail feather, and fork length as the difference in length between the tip of the innermost and outermost tail feathers. Both the right and left lengths of the fork were measured, and we computed the absolute difference in length as an estimate of fork fluctuating asymmetry. Swifts have very short tarsi and thus, as an alternative measure of skeletal size, we measured their sternum length with a caliper to the nearest 0.1mm. With each capture of an adult, the individuals are weighed with a scale at the nearest 0.1 g, and we counted the number of ectoparasitic louse-flies in their plumage. Alpine swifts are heavily infested by the hematophagous louse-fly *Crataerina melbae* (Diptera, Hippoboscidae) that has been previously demonstrated to affect nestling growth (Bize et al., 2003; Bize et al., 2004a) and adult reproductive success (Bize et al., 2004b). When infested by blood-sucking insects, hosts can defend themselves with cutaneous immune responses that make the biting site unfavorable for feeding by hematophagous insects and can cause damage to the parasite tissue (Wikel, 1996; Wikel, 1999; Owen et al., 2010). In agreement with this, the cutaneous immune response of Alpine swifts was found to affect the survival and blood meal size of the louse-flies (Bize et al., 2008b). To assess the parasite burden of the swifts, we counted the number of louse-flies in adult plumage.

Hence, the 11 phenotypic traits collected in this study can be grouped into three different broad categories, namely, biometric traits (wing, tail, fork, fork asymmetry, sternum, body mass), parasite load which could reflect the cutaneous immunity of the

bird, and reproductive traits (laying date, clutch size, brood size at hatching, brood size at fledging).

## Statistical analyses

All the analyses were performed using R version 4.1.2 (R Core Team, 2022). The birds used in this study ranged between 1 to 22 years of age. The data include repeated measurements for each individual throughout its breeding life (mean ± SE breeding observations per individual: 3.8 ± 0.1; 1st and 3rd quantiles: 1 and 5 observations per individual). At both ends of our age spectrum, we only had 2 observations of breeding birds at 1 year and 9 individuals with 14 observations at 19 years of age and older. To avoid biases caused by extreme values and thus improve the robustness of our results, we pooled observations at 1 year of age with those at 2 years of age and we pooled together in the same age category all the observations at 19 years of age and over (following Froy et al., 2013; Fay et al., 2021).

First, to tease apart changes in the phenotype driven by age-related effects, selective disappearance, and terminal illness or investment (van de Pol and Verhulst, 2006; Froy et al., 2013), we restricted our analyses to breeding adults (N = 244 males and 307 females) whose age was known (i.e. ringed at nestling or at one year of age, when still having different plumage characteristics than adults) and for which we had complete life history (i.e. followed from their first reproduction to death). We assigned a lifespan to individuals found dead or not seen since at least 2020 (i.e. not captured for at least 2 consecutive years). We used a mixed model approach that follows *equation (1)* in van de Pol and Verhulst (2006) which allows teasing apart within-individual age effects (experience, senescence) from demographic effects (selective appearance and disappearance):

$$r_{ij} = \beta_0 + \beta_W \times age_{ij} + \beta_1 \times AFR_i + \beta_2 \times Lifespan_i + u_{0i} + e_{0ij}$$

which is a two-level random intercept model with individual $i$, measurement $j$, random intercept term $u_{0i}$, residual error term $e_{0ij}$, within-individual change ($\beta_W \times age_{ij}$), selective appearance effect ($\beta_1 \times AFR_i$) and selective disappearance ($\beta_2 \times Lifespan_i$). By modeling the age of individuals as a continuous variable that is not centered on a mean individual age (i.e. $age_{ij}$), our models test for an absolute effect of age (i.e. chronological effect associated with a general pattern of senescence) rather than a relative effect of age (i.e. biological effect with individual deviation from the general pattern of senescence) (see Fay et al., 2021).

In all our mixed models, we also included a two-level fixed factor 'last observation' (last breeding observation = 1, previous breeding observation = 0) to test for terminal effects (terminal investment vs. terminal illness) that can mask the effect of senescence (Bouwhuis et al., 2009; Froy et al., 2013), and a two-level fixed factor 'colony' (Biel, Solothurn) to control for possible differences between breeding sites. We included individual ring and year of measurement as random factors to control for pseudo-replication and temporal heterogeneity. Parasite load varied with the date of capture following a quadratic curve (Bize et al., 2003), so we controlled for this variation by including linear and quadratic terms for the day of capture in the model testing the age-related variation of parasite load. Similarly, when analyzing body mass, we also controlled in our models for quadratic variations in mass in relation to the hour of the day and to the day of capture during the breeding season[1]. As we considered individuals from their first reproduction, individuals included in the analyses were already of adult size. Although sternum length is fixed in adulthood (no within-individual variation), we nevertheless kept this trait as dependent variable in our analyses as it may still show between-individual variation (e.g. selective disappearance of smaller individuals).

To identify first the variation of the 11 phenotypic traits with age in our population, we compared a series of mixed effects models separately for each trait and for male and female swifts. We tested models without any effect of age, with age as a linear effect, age as a quadratic effect, and age with different breakpoints known as threshold models (following Berman et al., 2009). We tested a single threshold varying between 4 to 12 years, and double thresholds one varying between 4 to 12 years and a second between 8 and 16 with at least 3 years intervals between the two. We compared these models using maximum likelihood (ML) estimation and identified the models that best described the variation with age of the phenotypic traits according to the Akaike Information Criterion (AIC). For each trait and each sex, we selected the models with ΔAIC<2 (Burnham and Anderson, 2002). If several models were selected, we used a model averaging approach (following Froy et al., 2013) using the R package MuMIn (Bartoń, 2011) to obtain the coefficient estimates of the co-variables in the models and assess the between individual effects (selective appearance and disappearance) and terminal effects for each trait and sex.

For the traits where we detected variation with age late in life, we then carried a second set of analyses to formally test for decline in late life (i.e. senescence), and whether this decline differed between sexes. From the best models (with ΔAIC<2), we calculated for each trait and sex the mean age of each threshold. We then built trait-specific 'late' dataset by pooling observations from both sexes made after the onset of senescence (i.e. using sex and trait specific thresholds). We considered the age at the second threshold as the potential onset of senescence for traits with two thresholds. We investigated sex-specific senescence using linear mixed models with age as linear effect to estimate the slope after the threshold and testing the interaction between age and sex. In these models, we also included individual ring and year of measurement as random factors, and we kept as fixed effect the variables selected from our previous approach using model averaging. In this second set of analyses, we increased our sample sizes at late age by taking into account data from individuals (N =

1 Dumas, M. N., St Lawrence, S., Masoero, G., Bize, P., and Martin, J. G. A. Variation in adult body mass is heritable, positively genetically correlated and under antagonistic selection between the sexes in a bird with little apparent sexual dimorphism. J. Anim. Ecol.

319 males and 391 females) whose age and lifespan were known, but for which we did not know their age at first reproduction, as they started reproducing before the start of our individual monitoring (before 2000). Nestling Alpine swifts in the colonies Biel and Solothurn have been ringed since at least 1968, and thus most individuals were of known age at the start of this individual monitoring. To compare rates of senescence across phenotypic traits, we scaled (i.e. standardized to zero mean and unit variance) the traits prior to the analyses.

As recommended by Schielzeth et al. (2020), we fitted *lmer* models (Gaussian distribution) to all studied traits to allow biologically meaningful comparisons of estimates across models even if not all the traits followed a Gaussian distribution. These authors showed that mixed models provide robust estimates of fixed effects even when the distributional assumption is strongly violated. Sample sizes may differ between models and traits due to missing values for some traits or some individuals.

# Results

## Age-related variation of trait

Our comparison of models showed that age-related variations in our phenotypic traits were usually best modelled with threshold models rather than quadratic models (See AIC table presented in appendix, Table A2). Results of the best models presented in Figure 1 show an age-related variation in all traits studied, at the exception of fork fluctuating asymmetry (Figure 1D), sternum length (Figure 1E), and body mass (Figure 1F). Models with 2 thresholds best described the predominant pattern of aging, as observed in both sexes for wing length (Figure 1A), tail length (Figure 1B), fork length (Figure 1C), parasite load (Figure 1G), laying date (Figure 1H), clutch size (Figure 1I), brood size at hatching (Figure 1J) and fledging (Figure 1K). Among these traits, the length of the wing, tail, fork, the clutch size, brood size at hatching and fledging showed an increase in both males and females early in life, until the first or second threshold, followed by a decline in at least one of the two sexes. Parasite burden and laying date show the opposite pattern with a decrease early in life, corresponding to a decrease in parasite load and an advancement of the laying date in the early reproductive years (Figures 1G, H).

## Selective appearance, selective disappearance and terminal effects

Estimates of selective appearance, selective disappearance and terminal effects derived from model averaging of the best models are presented in the Figures 2A–C. We found evidence of selective appearance (i.e. measured by including AFR) for male fork length and fluctuating asymmetry and in female brood size at hatching and fledging (Figure 2A). Males with longer and more symmetrical fork start to reproduce earlier. Females that tend to initiate their reproduction late in life tend to have lower brood size at hatching and fledging.

We found evidence of selective disappearance (i.e. effect of lifespan) for male tail length (Figure 2B), with males with longer tails being shorter lived and gradually disappearing from older age categories. We also observe a trend for selective disappearance of females of lower body mass (Figure 2B).

Finally, we found support for terminal effects (i.e. last year of observation) on male fork fluctuating asymmetry, sternum length, and brood size at hatching (Figure 2C). In their last year of life, males produced more offspring at hatching, and they also show an increase in fork asymmetry and a decrease in sternum length. Males also tend to show a decrease in body mass the last year before death (Figure 2C; Table A3 in appendix).

## Onset of senescence and late life rates of aging

For all the traits showing a variation with age, we then looked at age-related variation late in life (i.e. after the last threshold) to formally test for the presence of individual senescence. We found evidence of a decline with age late in life for 4 of the 11 traits (i.e. wing, tail, brood size at hatching, brood size at fledging; Figure 3). This decline was different between males and females for tail length, brood size at hatching and at fledging (Figure 3). Senescence rates for tail length were higher in females than in males, and senescence on the number of offspring at hatching and fledging were only visible in females.

Finally, threshold estimates presented in Figure 1 show asynchrony in the age at onset of senescence (i.e. second threshold) between sexes, with this onset being earlier in males for tail length and parasite load (Figures 1G, H).

# Discussion

Our analysis of age-related variation in eleven phenotypic and fitness-related traits in the long-lived Alpine swift shows evidence for variation in adulthood for all traits studied except sternum length, body mass and fork fluctuating asymmetry. Sternum length is a skeletal feature that is not expected to change once birds reach their final adult size (i.e., just prior to fledging in the Alpine swift; Bize et al., 2006b), and thus the lack of within-individual variation in sternum length in adulthood was expected. Although the absence of variation with age for the body mass and fluctuating asymmetry of the fork suggests a relatively stable body condition in this bird, we observe a tendency of decrease of body mass and detected an increased asymmetry of the fork in males the last year before death. In females, a senescence in body mass might be masked in our study at the population level by the selective disappearance of individuals with lower mass. The variation with age of the other traits was best described by segment models with 2 thresholds, such that traits show an increase early in life followed by a plateau and/or

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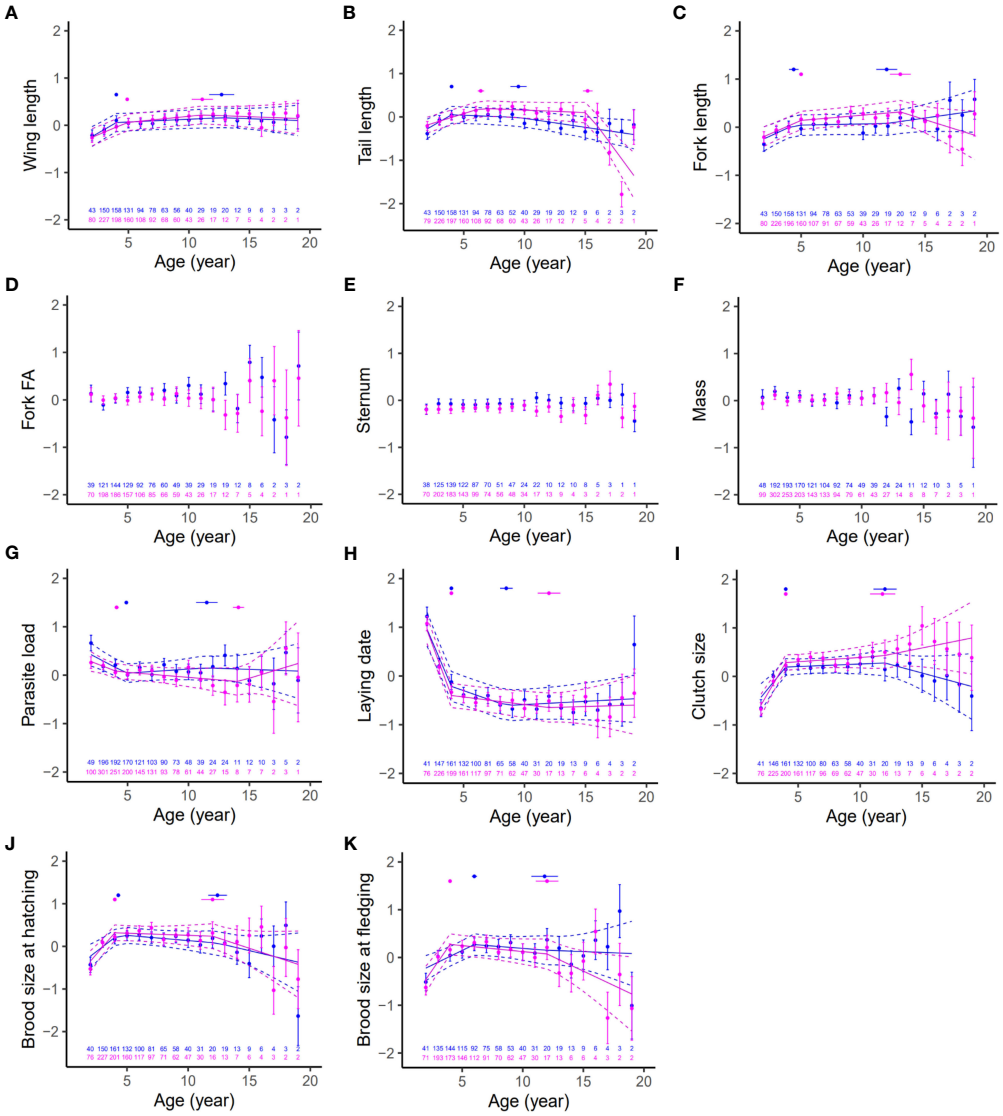


FIGURE 1
Age-related variation in biometric traits, parasite burden, and reproductive traits in male (blue) and female (pink) Alpine swifts. Traits (Y-axis) are scaled and centered and age (X-axis) is expressed in years, allowing for an immediate comparison across traits and between sexes of rates of change with age. The graphs show the segments obtained from the predictions of the threshold models with 95% CI (dashed lines) and the mean value of the trait at each age (mean ± SE in blue for the males and pink for the females). The horizontal lines above the segments show the range of variation of the thresholds for each sex calculated from the models with ΔAIC<2 (mean thresholds ± SE). If the "null" model with no age-related variation was retained by our model selection, we did not plot the segments from the prediction of the thresholds model (i.e. fork FA, sternum, mass). For each of the traits, sample sizes at a given age are indicated at the bottom of each panel in blue for the males and pink for the females.

decline later in life. This 'bell-shape' pattern is consistent with what has been observed in numerous species (Catry et al., 2006; Lecomte et al., 2010; Frankish et al., 2020; Saraux and Chiaradia, 2022). In the Alpine swifts, most traits showed increase or improvement until 4 to 12 years of age (e.g., increased fork length, decreased parasite load, or earlier laying date) followed by a decline at older ages. Such widespread evidence of trait improvement over the first year of breeding is striking, and the mechanisms explaining improvement with age (e.g. appearance or disappearance of certain phenotypes;. constraint vs restraint hypotheses; Forslund and Pärt, 1995; Saraux and Chiaradia, 2022), remain to be investigated in detail in this species.

## Selective appearance, selective disappearance and terminal effects

We used a statistical approach to separate within-individual effects (aging per se) from demographic effects (van de Pol and Verhulst, 2006; Fay et al., 2021). This approach is important as we found evidence of demographic effects on age-dependent trait variation. Interestingly, we found selective appearance of males with shorter fork and a trend for males with higher asymmetry of their fork. Hence, males with longer and symmetrical fork start reproducing earlier. The length and symmetry of the fork could thus reflect the quality of a male and be involved in its mating success.

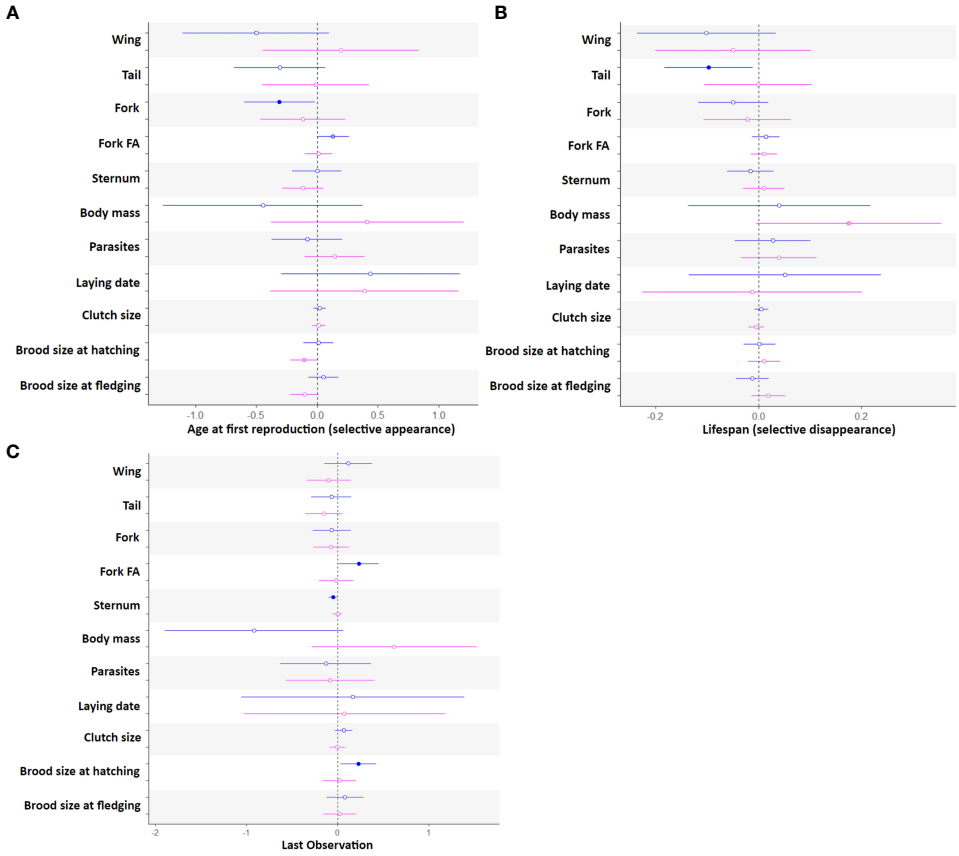


FIGURE 2
**(A–C)** Estimates with 95% confidence for selective appearance **(A)**, selective disappearance **(B)** and terminal effects **(C)** in female (pink) and male (blue) Alpine swifts. The significance of each estimate is indicated by filled circle when significant ($P < 0.05$), by lightly colored circles when marginally significant ($0.05 \leq P \leq 0.06$), and by open circles when non-significant ($P<0.06$).

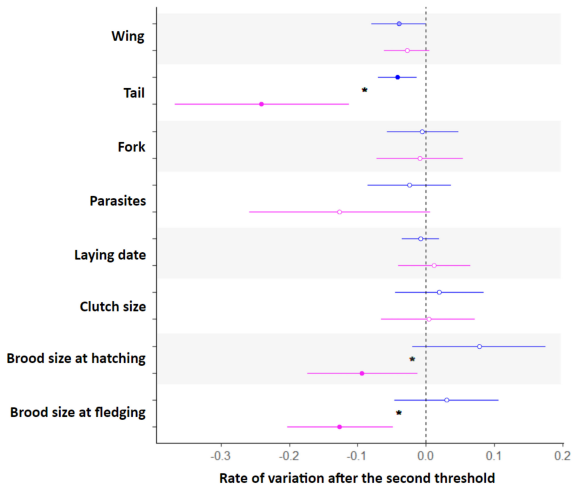


FIGURE 3
Estimates of rates of ageing with 95% confidence in female (pink) and male (blue) Alpine swifts. The significance of each estimate is indicated by a filled circle when significant ($P < 0.05$), by lightly colored circles when marginally significant ($0.05 \leq P \leq 0.06$), and by open circles when non-significant ($P < 0.06$). Significant differences in the rate of ageing between males and females for a given trait are indicated by a star symbol. The traits with no evidence of age-related variation (i.e. sternum, fork fluctuating asymmetry, body mass) were removed from these analyses.

This result brings support to a previous phylogenetic comparative analyses suggesting that fork depth is under sexual selection in swifts (Hasegawa and Arai, 2020). In females, we observed a trend for selective appearance of females with lower reproductive performance. Females that start reproducing earlier show higher brood size at hatching and fledging. Therefore, the selective appearance of males with shorter fork and females with lower reproductive performance further supports the strong increase of these traits observed early in life. At older ages, we observed the selective disappearance of males with longer tails and a tendency for females with lower body mass. The latter might be related to the general quality of females suggesting that higher quality females might have a higher longevity (Weladji et al., 2006; Tettamanti et al., 2015). This result could mask a potential decline of body mass in females at older ages in our study.

We also found terminal effects, as an increased brood size at hatching the last year before death in males. However, we cannot know if the death of the males after this breeding season is the consequence of the increased reproduction or the cause. The increased brood size the last year of life supports a terminal investment of males during incubation. Another possible explanation could be that males, before their death, mate with younger females increasing the performance of their last

reproduction. However, this increased reproduction can also involve high costs and therefore be the cause of death, via a trade-off between allocation of energy for reproduction at the expense of survival (Stearns, 1992). This is also consistent with the trend observed in males for a terminal illness with a decrease of body mass and the increase of fork asymmetry the last year before death.

## Age-related changes in flight-related biometric traits

The Alpine swift is a very aerial bird that can remain in flight without interruption for over 200 days (Liechti et al., 2013). Our results show that biometric traits associated with flight, namely wing and tail, vary throughout adult life, with an increase in size until 4 to 6 years of age followed by a decline after 9 to 15 years of age. In particular, tail length showed strong senescence in both sexes, although the decrease in males might be partly driven by selective disappearance of individuals with longer tails. The decline was moderate for wing length in both sexes. These results are qualitatively comparable to the patterns of variation with age of morphological traits observed in the barn swallow (*Hirundo rustica*, Møller and de Lope, 1999). Feather abrasion over time (days to years) may explain the reduction in wing and tail length with age. However, since Alpine swifts molt every year their wing and tail feathers, the decrease of length observed with age might rather be explained by a decrease of feather growth rate with age, as recently evidenced in the central tail feathers of barn swallows (Adamkova et al., 2022). The decline in biometric traits late in life could have implications for flight ability in Alpine swifts since body mass and the length and shape of the wings, tail, and fork influence flight maneuverability and energy expenditure during flight in birds (Evans and Thomas, 1992; Thomas, 1993c; Norberg, 1995a). Swifts have long narrow wings and low wing load, which are biometric features that allow them to make sharp turns to capture insects (Norberg, 1995b). Hence, the shortening of tail and wing length with age in the Alpine swift could increase the cost of flight and decrease flight performance and foraging success at older ages, as observed in the grey-headed albatross (Catry et al., 2006). These decreases in flight performance with age could, in turn, have an impact on reproductive success in later life. However, in our study system, this hypothesis is only partially supported, because, although we found strong evidence of senescence for flight-related traits in both sexes, we only found evidence for reproductive senescence in females. Therefore, the senescence of reproductive traits in this species is probably not simply explained by changes in size and body condition alone, as in this case we would also expect to see a decline in reproductive parameters in males as well. The decline of biometric traits later in the life we observe in Alpine swift might also have an impact on their migration. This is even more likely since the ratio between the size of the bird, the length and shape of the wings and tail are associated with specific tactics of migration, which in this species for instance would favor energy-minimization (Norberg, 1995b). To confirm this hypothesis, it would be necessary to study the characteristics of migrations (Meier et al., 2020) after the onset of the senescence of biometric traits to test whether senescence impacts the distances traveled and/or the travel time to reach the breeding location. A study on barn swallows showed that tail and wing length decreased in older individuals, as did their migratory performance, resulting in a later arrival on the breeding site (Møller and de Lope, 1999).

## Age-related changes in parasite load and immunosenescence

Parasites derive their resources from their hosts, and hosts exposed to parasites may therefore incur fitness costs (Møller et al., 2009a). Hosts can, however, defend themselves against parasites by mounting immune responses (Wikel, 1996; Wikel, 1999; Owen et al., 2010), which can limit the amount of resources extracted by hosts and their infestation load. Hence, one hypothesis is that the decline of the immune responses in old age leads to greater exposure to parasites (Møller and de Lope, 1999; Hayward et al., 2015; Peters et al., 2019) and, in turn, a decline in host performance in old age. Alpine swifts are heavily infested by blood-sucking louse-flies that are known to negatively impact the reproduction of this bird species (Bize et al., 2003; Bize et al., 2004a; Bize et al., 2004b; Bize et al., 2005), and a previous study showed that the cutaneous immune response of swifts plays an important role to defend themselves against louse-flies (Bize et al., 2008b). We found no evidence that parasite load differed between the sexes or increased with increasing age. The lack of sex difference in parasite load in adult swifts is similar to what has been found in nestlings where males and females show similar levels of parasite infestation and cell-mediated immunity (Bize et al., 2005). Overall, our results are similar to those of a recent study on the common tern (*Sterna hirundo*), a long-lived seabird, which found no difference between males and females in innate immune parameters and no clear senescence of these parameters (Bichet et al., 2022). In Seychelles warblers (*Acrocephalus sechellensis*) as well, the infection of a blood parasite does not increase in older birds, providing no evidence for immunosenescence (Hammers et al., 2016). Also, recent meta-analyses did not find evidence of differences between the sexes in immunity and immunosenescence in wild populations (Kelly et al., 2018; Peters et al., 2019). Hence, our results provide little support for immunosenescence as an important driver of reproductive or biometric trait senescence in the Alpine swift. However, studies with direct measures of the immune system and greater diversity of measures of exposure to parasites (such as blood meal size) (Bize et al., 2008b) are needed to gain greater insights on immunosenescence in this host-parasite system.

## Age-related changes in reproductive traits

Our results show that, as observed in other vertebrates in the wild (Lemaître and Gaillard, 2017; Cooper et al., 2021), the reproductive performances of Alpine swifts first increased with age over the first reproductive attempts for both males and females, a finding that can is often attributed to birds gaining breeding

experience early in life (Forslund and Pärt, 1995; Saraux and Chiaradia, 2022). However, there were marked differences between the sexes later in life as we detected evidence of reproductive senescence in females that was not observed in males. That is, after reaching a peak, females showed a decline in reproductive performance later in life, with strong evidence for senescence in brood size at hatching and fledging from around 12 years. In males, egg-laying date advances in the early reproductive years, and clutch size, brood size at hatching and fledging increase before reaching a plateau at older ages. Note that since males do not lay eggs, evidence of male age-related variation in laying date and clutch size is likely to reflect selection or differential allocation by females based on male age and reproductive experience (Komers and Dhindsa, 1989; Michl et al., 2005; Segami et al., 2021). That is, females may lessen their allocation into reproduction (delay breeding and produce fewer eggs) when mating with young and inexperienced males compared to old and experienced males. Altogether, these results indicate stronger signs of reproductive senescence in females compared to males in the long-lived Alpine swift. Our results are consistent with observations in wild Soay sheep, with a decline in reproductive performance in females contrary to an increase of reproductive performance in males which then plateaus (Hayward et al., 2015). However, this contrasts with findings in the wandering albatross which is a long-lived and monogamous bird like the Alpine swift, with similar parental roles between males and females (Pardo et al., 2013). The breeding probabilities of males albatross decrease at a faster rate than in females (Pardo et al., 2013).

Contrary to polygynous and highly dimorphic species, with sex-specific allocation to sexual signaling and mating, it is less evident to identify potential causes of these sex-specific patterns of reproductive senescence in monogamous and monomorphic species. In the great tit (*Parus major*), it was found that half of the decline in reproductive success is related to reduction in brood size at hatching and fledging, and thus to egg failure and nestling mortality; clutch size did not contribute to senescence of recruit production (Bouwhuis et al., 2009). The authors of this study suggested potential proximate mechanisms underlying the senescence in reproductive success, such as incubation behavior, the composition and quality of eggs, or a decline of male fertilization efficiency and sperm quality as reviewed also in Lemaître and Gaillard (2017). In our study population, however, results show no senescence of male reproductive traits and even an improvement in the last breeding attempt before death. It is thus unlikely that male fertility and sperm quality decrease with age in the Alpine swift, as opposed to other wild birds (e.g. Møller et al., 2009b). Another explanation could be differences in energy expenditure between males and females during reproduction. Although males, as well as females, do incubate the clutch and provision the brood, the production of eggs by females is energetically demanding and can lead to costs as reviewed by Williams (2005). For example, in a wild population of lesser black-backed gulls (*Larus fuscus*), experiments where females were forced to lay extra eggs show consequences not only on the quality of the extra eggs (Nager et al., 2000), but also females' rearing capacity (Monaghan et al., 1998) and their local return rates (Nager et al., 2001). Hence, Alpine swift females may have greater reproductive costs than males due to egg production, which in the long run might explain the decline in reproductive traits observed in females as opposed to males in our population. Furthermore, although Martins and Wright (1993) reported in the closely related common swift that males and females show similar levels of self-feeding (Martins and Wright, 1993), and by extension self-maintenance, it cannot be excluded that males and females' life history strategies can evolve with age, as seen in the wandering albatross (Lecomte et al., 2010). In this species, the decline in reproductive performance suggests that older males allocate more in self-maintenance as opposed to reproduction compared to young males (Lecomte et al., 2010). Insights on sex and age-related variation on the energetics and foraging behaviors in the Alpine swifts will be required to answer these questions. Furthermore, to better understand the mechanisms behind variation in reproductive senescence between males and females, it would be interesting to investigate other physiological traits, especially related to reproduction in females. For example, it has been suggested that the deregulation with age of the pituitary-hypothalamic-ovarian axis, as a consequence of a somatic deterioration, could be an important driver of reproductive senescence in females (Lemaître and Gaillard, 2017). How males and females differ in the regulation of their oxidative balance could also be an interesting lead in the Alpine swift, as previous research in this species showed senescence in cell resistance to oxidative stress (Bize et al., 2014) as well as sex-specific covariation between resistance to oxidative stress and fecundity and survival (Bize et al., 2008a). Males who survived to the next breeding season tended to be more resistant to oxidative stress, and females with higher resistance to oxidative stress laid larger clutches (Bize et al., 2008a). In our population, reproductive senescence might thus be due to global physiological decline with age, associated with costs of reproduction, and/or to an allocation later in life in somatic functions responsible for longer lifespan as hypothesized above.

Finally, sexual selection by females for males that are more resistant to aging, especially at older ages, or by males for females that are more prone to allocate in reproduction, could also partially account for the difference in senescence patterns between males and females in our population (Promislow, 2003). Although there is currently little information on sexual selection in the Alpine swift[2] phylogenetic comparative analyses are suggesting that fork depth is under sexual selection in swifts (Hasegawa and Arai, 2020).

## Asynchrony in senescence across traits

Our results support the growing evidence that asynchrony in senescence between traits is a common pattern in wild animals (Bouwhuis et al., 2012; Nussey et al., 2013; Hayward et al., 2015; Bouwhuis and Vedder, 2017; Cooper et al., 2021; Fay et al., 2021). In

2 Dumas, M. N., St Lawrence, S., Masoero, G., Bize, P., and Martin, J. G. A. Variation in adult body mass is heritable, positively genetically correlated and under antagonistic selection between the sexes in a bird with little apparent sexual dimorphism. J. Anim. Ecol.

the Alpine swifts, tail length started to senescence earlier in males (~9 years) than in females (~15 years) and presented faster rate of senescence than other biometric traits. Among the different tail shapes of birds, forked tails, as for example in swifts and swallows, are more vulnerable to damage (Thomas, 1997). Moreover, aerodynamic performances of birds are more sensitive to a variation of the shape of the wings than the tail (Thomas, 1997). Thus, a decrease in tail length would have a lower impact on flight performances compared to a decrease in wing length. From these observations, and because the tail of birds also shows higher fluctuating asymmetry than the wings, Thomas (1997) suggested that natural selection might act differently on the tail and wings. This assumption could also partly explain the higher senescence of the tail observed in the Alpine swift, compared to the wings. The length of the tail might not be subjected to strong selection for better resistance, and neither would benefit for a selection in greater allocation in self-maintenance to preserve its length.

Regarding reproductive traits that show senescence in females, it is interesting to note that the effects of senescence are becoming increasingly evident as allocation to reproduction progresses, from no detectable effects on egg laying and clutch size to detectable effects on brood size at hatching and fledging. It suggests that reproductive senescence in females might be linked to an accumulation of costs of reproduction, from egg laying to incubation and food provisioning. Our results are in line with the study of Bouwhuis et al. (2009) on female great tits, which found that the age at peak of performance occurs earlier for the number of fledglings than the clutch size, and that the number of fledglings declines post-peak contrary to the clutch size. However, the opposite pattern was observed in the White-tailed ptarmigan (*Lagopus leucurus*) (Wiebe and Martin, 1998). Individuals from the oldest age classes showed senescence of early breeding stage traits (lay date, clutch size) but showed the highest renesting rate and fledging success. The identification of traits that senesce at faster rates within and across species may provide a valuable starting point to gain insights on the main mechanisms of aging regulating changes in morphology, physical performance, and reproductive success.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: Zenodo, Available at: https://doi.org/10.5281/zenodo.8329067.

## Ethics statement

The animal study was reviewed and approved by Swiss Federal Agency of Environment, Forests and Landscapes (project number BE[72] and SO[72]).

## Author contributions

PB and SR designed the study. PB carried out the field work. HM and PB carried out the statistical analyses. HM drafted the manuscript and all authors revised it. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.983266/full#supplementary-material

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EDITED BY
Jean-Michel Gaillard,
Université Claude Bernard Lyon 1, France

REVIEWED BY
Sophie Beltran-Bech,
UMR7267 Ecologie et biologie des interactions (EBI), France
Claudia Fricke,
Martin Luther University of Halle-Wittenberg, Germany

*CORRESPONDENCE
Thomas Tully
thomas.tully@sorbonne-universite.fr

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# Diversity, plasticity and asynchrony of actuarial and reproductive senescence in the Collembola *Folsomia candida* (Willem, 1902)

Thomas Tully*

Sorbonne Université, UPEC, CNRS, IRD, INRA, Institute of Ecology and Environmental Sciences - Paris, IEES, Paris, France

Flourishing recent comparative studies on senescence have revealed an uncovered diversity across the tree of life of the shapes of the age trajectories of mortality (actuarial senescence) and to a lesser extent of reproduction (reproductive senescence). Evolutionary theories have been called up to explain why some species suffer from positive senescence while others benefit from negligible or even negative senescence. We still know little about how, within a species, the shapes of the age trajectories of different traits are linked to each other and how they vary or covary depending on the genetic background and environmental conditions. We report here the results of an experimental study whose aim was to describe the actuarial and reproductive senescence in various genetically distinct lineages of a Collembola, a hexapod with indeterminate growth. We compared the age trajectories of individuals raised under two food regimes to study if and how the shapes of these age trajectories are plastically modified by environmental conditions. We found clear evidence of actuarial and reproductive senescence, especially when the springtails were fully fed. Clutch size increased as female become older and then declined progressively after reproduction reached a maximum. This age decline in fertility went along with a progressive slowing down of the pace of the egg-laying, a reduction of egg quality (more sterile eggs), while egg size undergoes little change with age. We found that the onset of reproductive decline occurred before the beginning of actuarial senescence, and show that escaping senescence is physiologically possible for certain lineages under dietary restricted conditions.

KEYWORDS

**age trajectory, ageing, phenotypic plasticity, dietary restriction, egg size, negligible senescence, lifespan, indeterminate growth**

## Introduction

While ageing affects every organism - because all become older with time - only some of them senesce. Indeed, following several authors (Medawar, 1952; Baudisch, 2011), we use the word ageing to describe the effect of time on adult's phenotypes without its familiar connotation of decline at advanced ages while senescence refers to a progressing age-related decline of several functional traits.

Empirical studies on senescence in the wild, and on experimental populations, have focused on two major fitness components: survival (actuarial senescence) and reproduction (reproductive senescence). While it has long been thought that senescence was somehow inevitable – apart from a few well-known special cases (Martínez, 1998; Schaible et al., 2015) – survival and reproduction have been found to decrease at various rates, depending on species, populations or on some environmental factors. Several comparative studies have demonstrated the existence of a great diversity in the patterns of ageing, the shape of the reproductive and survival age trajectories ranging from senescence (decline of reproduction or survival with age) to negative senescence (increase of reproduction or survival with age) through negligible senescence (negligible effect of age) (Baudisch, 2011; Baudisch et al., 2013; Jones et al., 2014; Baudisch and Stott, 2019; Colchero et al., 2019; Lemaître et al., 2020; Reinke et al., 2022).

Similarly, some studies have revealed a remarkable qualitative intraspecific variation in the age pattern of reproduction and mortality in species like dogs (Kraus et al., 2013), social insects (Münch et al., 2008), reptiles and amphibians (Cayuela et al., 2020a; Tully et al., 2020; Cayuela et al., 2021) or hexapods (Mallard et al., 2015; Li and Zhang, 2021), while other studies have found similar age-specific mortality patterns across populations of the same species (Reichard, 2016).

Although many traits can be simultaneously affected by senescence, most studies have focused on describing the effects of age on a single trait in isolation, usually survival rate (Hoekstra et al., 2020). Few longitudinal studies have jointly studied for instance the shape of the age trajectories of mortality and reproduction (Roach and Carey, 2014; Mallard et al., 2015; Tully et al., 2020; Li and Zhang, 2021). A multi-trait approach is, however, required to describe and compare how the effects of age manifest themselves on the different components of fitness. This can help address the question of the timing of senescence and more particularly whether it is a synchronized process. Indeed, in theory, the onsets of actuarial and of the different components of reproductive senescence should be close to each other, senescence being expected to begin at the time of reproductive maturation (Williams, 1957). This prediction has been challenged (Gaillard and Lemaître, 2017). Several empirical studies have for instance reported pronounced levels of asynchrony between actuarial and several components of reproductive senescence. The death rate of green whip snakes has been shown to increase after about six years while their fecundity continued to increase with age (Cayuela et al., 2020b). Similarly, in a population of meadow vipers, the mortality was found to increase markedly with age after about 4–5 years while no sign of reproductive senescence could be observed on several reproductive traits (Tully et al., 2020). In the laboratory, a cohort follow-up of the Collembola *Folsomia candida* showed that mortality started to increase progressively long before reproduction began to decline (Mallard et al., 2015). These observations are still too few in number to know to what extent this asynchrony is general and whether reproductive decline is always subsequent to the onset of actuarial senescence. On the other hand, the synchrony of the different components of reproduction remains very little studied (Tully et al., 2020).

The timing and rate of senescence have also been found to vary between groups of individuals such as sexes, populations, lineages, and according to environmental factor such as temperature, competition, food provisioning for instance (Massot et al., 2011; Kelly et al., 2013; Hayward et al., 2015; Hayward et al., 2015; Mallard et al., 2015; Stroustrup et al., 2016; Gribble et al., 2018; Cayuela et al., 2020b; Tully et al., 2020; Cayuela et al., 2021).

Food provisioning is probably the most studied environmental factors known to modulate the timing and intensity of senescence: a moderate reduction of food intake through caloric or dietary restriction increases the longevity in a large range of taxa by delaying the onset and slowing down the rate of actuarial senescence (Mair and Dillin, 2008). Quite logically, low food availability is also generally associated with a reduced reproduction given that reproductive output is constrained by the availability of nutrients. The effect of a food scarcity on reproductive senescence has been less well studied (Auld and Henkel, 2014). In a freshwater snail, a change in diet (Lettuce compared to *Spirulina*) associated with a longer somatic lifespan was tied with a prolonged reproductive lifespan (Auld, 2018) and the negative effect of parental age on offspring survival was stronger in individuals fed with richer food (*Spirulina*), thus suggesting that, as for mortality, a poor diet (Lettuce) may result in slowing down reproductive senescence. Similarly, in the Collembola *Folsomia candida*, compared to a rich diet (yeast), a poor diet (leaf diet) decreased the oviposition rate and also delayed and slowed down the loss of mean fertility as groups of individuals become old (Van Amelsvoort and Usher, 1989). In a selection experiment, the shape of the age-specific fecundity of *Drosophila melanogaster* changed depending on the food selection regime (Dasgupta et al., 2022). Dietary restriction has also been found to slow down the rate of germline stem cells loss with age in *Drosophila melanogaster* (Mair et al., 2010).

These studies suggest that reproductive senescence may itself be modulated by food provisioning but the specific analysis of how a change in food provisioning modifies the shape of the reproductive age-trajectories remains to be clarified. Can dietary restriction modify the mean level of reproductive output and also, on the long term, the onset and rate of reproductive senescence? Are the age trajectories of different components of reproduction equally sensitive to a change in food provisioning? And finally, can an environmental factor like food modulate the asynchrony between different components of ageing?

Our aim here is to use a laboratory longitudinal study of the ametabolous hexapod *Folsomia candida* Willem, 1902 (Collembola: Isotomidae (Willem, 1902)) to address these various questions. This species has already proven to be an interesting model organism in ageing research (Van Amelsvoort and Usher, 1989; Tully and Ferrière, 2008; Tully and Lambert, 2011; Mallard et al., 2015). Being a parthenogenetic species, individuals - all females - can be isolated, raised and followed up from birth to death, which usually

occurs after several months. Moreover, Collembola are in a way comparable to non-avian reptiles and amphibians that have been shown to be fascinating groups to study ageing (Reinke et al., 2022), in that, unlike most hexapods, they are iteroparous indeterminate growers: they continue to mould and grow up to an asymptotic size after reproductive maturity and they reproduce multiple times during their lifespan (Briti, 1951). Moreover, their fecundity usually increases with size. In *F. candida*, the size of the first clutch increases linearly with the body weight at maturity (Stam et al., 1996). In several species of Collembola, including *F. candida*, fecundity increases right after maturation as the adults become old and continue to grow but not after their asymptotic size is reached (Gregoire-Wibo, 1974; Grimnes and Snider, 1981; Booth, 1983; Snider, 1983). We report in Figure S1, the detailed relationship between clutch size and adult body length which shows that body size determines the clutch size in *F. candida*, at least during their lives as young adults. *F. candida* is thus a good candidate species for expressing slow or negligible senescence given that it is known that negligible or negative senescence can evolve in species that continue to grow after maturity and that gain reproductive capacity as they become older and thus also larger (Vaupel et al., 2004; Baudisch, 2008).

Below, we list the questions that we will address in this work. First, does this species suffer from actuarial and reproductive senescence or can it benefit from negligible senescence as one might predict? We will describe the shape of the long-term age trajectories of mortality and several components of reproduction, namely clutch size, inter-clutch intervals, egg size and proportion of sterile eggs. Second, to what extent is the senescence of the different components of fitness synchronized? To address this question, we will compare the timing of actuarial and reproductive senescence and the timing of senescence of the different traits that make up reproduction. Third, what is the degree of plasticity of these age trajectories? By following the life history trajectory of isolated springtails either fed without limitation or dietary restricted, we intend to describe and compare the environmentally induced malleability of the age trajectories of mortality and reproduction. More specifically we wish to verify the expected protective effect of dietary restriction on longevity and mortality and explore the potential effects of food deprivation on how various components of reproduction may change with age. Finally, how genetically variable are these age trajectories? We will compare several genetically distinct lineages of Collembola to explore if and how the age trajectories of mortality and reproduction and the aforementioned issues can genetically vary within a species.

# Materials and methods

## Model organism

We used, as a model organism, the Collembola *Folsomia candida*, which is a ~ 2mm long ametabolous unpigmented hexapod inhabiting in the wild microhabitats like moist layers of soil, decaying wood, leaf litter, edges of streams, and underground environments such as caves or mines (Fountain and Hopkin, 2005). The asymptotic body length (2 to ~2.5mm) can be reached in about two months at 15-16°C (Booth, 1983; Mallard et al., 2015; Mallard et al., 2019). Populations are composed of females who reproduce parthenogenetically and can form aggregations in the soil and in cultures (Usher and Hider, 1975). Population density can vary a lot in the wild between sites and over the seasons (Krivtsov et al., 2006). Also often absent or elusive, this species is sometime relatively abundant as in some caves (Alonso et al., 2019) and can even become dominant, reaching average densities of more than 500 individuals per 100g of dry forest litter (Krivtsov et al., 2003; Krivtsov et al., 2006). Being parthenogenetic, isolated individuals produce clonal populations that can be maintained relatively easily in the laboratory. The clonal lineages collected and kept in our laboratory come from two genetically distinct clades (Tully et al., 2006; Tully and Ferrière, 2008; Tully and Potapov, 2015) with contrasting life histories. We have reported large difference on several life-history traits among the two clades (called clade A and B). Collembola from clade B have on average a higher fecundity than those from clade A but also a higher mortality and shorter lifespan (Tully and Ferrière, 2008; Tully and Lambert, 2011). Collembola of clade B generally reach an asymptotic size smaller than those of clade A (Mallard et al., 2015; Mallard et al., 2019) which suggests the existence of genetic compensation between reproduction, growth and maintenance. Within each clade, the different lineages are more similar in terms of the studied life history traits, although some diversity still exists (Tully and Ferrière, 2008; Tully and Lambert, 2011). This springtail can reach a long lifespan (up to more than one year) in the protected laboratory conditions where mortality is low (Tully and Lambert, 2011; Mallard et al., 2015). We found that two strains from the two clades suffer from actuarial and reproductive senescence, but with especially contrasted patterns of age-dependent survival (Mallard et al., 2015).

## Experimental protocol and data collection

We studied and compared eleven isofemale clonal lineages of *F. candida* issued from the two above-mentioned phylogenetic distinct clades, labelled 'clade A' and 'clade B'. Clade A is composed of five lineages called "AP", "BR", "BV", "GB", "HA", and clade B of six, called "DK", "GM", "PB", "TO", "US" and "WI". The letters that designate these strains come from the initial sites of origin of these strains or from the people who collected them (Tully et al., 2006). These lineages come from eleven distinct geographical origins in Europe (France, Germany, The Netherland, Great Britan) and North America (Michigan, Wisconsin), with no clear phylogeographical pattern (Tully et al., 2006). Most of the reported genetic, life-history and morphological diversity was between the two clades (Tully et al., 2006; Tully and Ferrière, 2008; Tully and Potapov, 2015). For each of the eleven strains, we isolated 20 individuals right after birth. To avoid confounding "strains" effect with "culture population" or "clutch" effects, these individuals originated from at least four clutches coming from different replicates of stock populations that have been maintained in incubators at 21°C and 100% relative humidity for several months. The 220 individuals were kept isolated in rearing

containers (52 mm diameter and 65 mm high polyethylene vials filled with a 30 mm layer of plaster of Paris prepared with water mixed with 600 mL of Pébéo graphic Indian ink to darken it) and raised and followed up until death. They were kept in similar conditions during their whole lifespan (alone, 21°C, 100% relative humidity) except for food provisioning: for each strain, 10 individuals were maintained in high food conditions (food was provided ad libitum) and the 10 others in low food conditions. Food was provided as a small pellet (5uL) of a mixture of agar-agar and dried baker's yeast in a standardized concentration and volume (5000 mL water+80 mg agar+800 mg dried yeast, to produce pellets of 2 mL). In the low food condition, we applied an intermittent fasting regime by providing food one day per week, followed by six days of fasting. This was done for practical reasons because, given the Collembola size, it was not possible to control the amount of food eaten by varying the pellet size or concentration of yeast. Intermittent fasting was also chosen for biological reasons given that regular periods of starvation are normal for many invertebrates (Fontana and Partridge, 2015).

Rearing containers were visually inspected regularly for clutches. This monitoring allowed us to know when each female laid a new clutch, and thus to know the number of clutches laid by each individual, and to measure the interval between successive clutches.

For each of the 1347 clutches laid during this experiment, clutch size was measured by counting the eggs by eye under a dissecting microscope. We photographed about one third of the clutches (randomly selected) to measure the size of the eggs using a digital camera fixed on a dissecting microscope. More precisely we measured the diameter of 9834 eggs from 583 clutches, a couple of days after oviposition, before their chorion ruptured (Marshall and Kevan, 1962). We counted the number of sterile eggs (eggs that fail to hatch and whose chorion has not ruptured) in 404 randomly chosen clutches. Clutches were removed from the containers before egg hatching to keep females isolated during their whole lifespan. Digital pictures were taken weekly or so to measure the female length and track their growth trajectory until death. Body length was measured from the front of the head to the rear of the abdomen. Repeatability of the egg size and body length was measured in an independent experiment and reached 79% for egg size and 96% for body length (Tully and Ferrière, 2008).

During the 571 days of the experiment - the time it took for all the individuals to die - 8 individuals were lost or died accidentally due to improper handling, while the 212 others died 'naturally'.

We used the data collected to study the effect of age, food regime (ad libitum versus dietary restriction) and to search for genetic and environmentally induced variability on the following life-history traits: lifespan and survival rates, clutch size, inter-clutch intervals, egg size and proportion of sterile eggs.

## Statistical analysis

In the analysis, we systematically compared the four groups of Collembola made by the two clades raised under the two food regimes (clade A & B * ad libitum versus dietary restriction) and we used the same four colours to identify these groups in the different figures. Additionally, we report, for some traits, the variability among individuals and among clones within each clade. These graphs underline not only the mean age trajectories estimated at the level of a group of individuals but also the age trajectories measured at the level of each individual.

The analyses have been made with R (The R Development Core Team, 2021) and all the figures have been produced with the tools from the library *ggplot2* (Wickham, 2009) and *visreg* (Breheny and Burchett, 2017).

Analysing the complex non-linear age trajectories is not straightforward. We choose to fit models with non-linear smoothers, such as generalized additive models (*gam*), to describe the shapes of the life history age trajectories because these models do not need to specify *a priori* a specific shape that would rely on a linear effect of age on the studied traits. Comparing statistically the life-history age trajectories among the different groups is tricky given the potential complexity of the shapes of these trajectories and given the structure of the measurements (eggs nested within clutches, clutches within individuals, individuals within clones, clones within clades and clades within food regime…). Unfortunately using a single generalized additive mixed model (*gamm4*) to model our data, and thus statistically test for differences among clades or clones and treatments while taking into account different smoothed effects of age for each group of individuals and the random effects (multiple measurements per individual) was not possible due to convergence problems. Thus, for most traits, generalized additive models (*gam* from *mgcv* library) were used to fit a smooth trajectory on each group of individuals (clades*food regimes) independently and the predicted mean trajectories were plotted to reveal the mean shapes of the various age trajectories with minimal constraints (Wood, 2017). Their associated confidence bands can be used to determine by eye whether the trajectories differ, and if so, over which age range the divergences are most marked (Cumming, 2009). For some traits, we grouped the data collected by periods of 14 days (fortnights) or of longer periods and used generalized linear mixed models (*lmer* from *lme4* library) to estimate the mean and 95% confidence intervals of each of the four groups of individuals (clades*food) within each fortnight period. These models take into account, in their random parts, the structure of the data (eggs nested within clutches, clutches within individuals, individuals within clonal lineages) and thus provide reliable unbiased predictors. As for gams, the confidence intervals associated with the fortnights' means can be used to visually compare and discuss the differences between groups of these age trajectories. The results of the main statistical models selected to support our analyses are presented in detail the tables and in the Supplementary Materials.

### Life span and survival

We used Kaplan-Meyer survival curves – computed and plotted with the R *survival* (Therneau and Grambsch, 2000) and *ggsurvplot* libraries (Kassambara et al., 2021) – to compare visually and statistically (with log-rank tests) the lifespan distribution among food regimes for each clone (Figure 1) or among the four groups of clade and food regimes (Figure 2). We also used Cox proportional

hazard models (*coxph* function form *survival* library) to estimate and compare the median lifespans and hazard ratios (Table 1). The hazard functions h(t), which describe the risk of mortality per unit of time, have been estimated for each clade and food regime with the *bshazard* library (Rebora et al., 2018) which enables to produce nonparametric smoothed estimates of the effect of age on the instantaneous mortality rates (Figures 3, S4). We were constrained to group together the clones within each clade to have a sufficiently large number of longevities to estimate the hazard functions, after verifying that the survival curves of each clone did not vary much within each clade and food regime (Figure S2).

## Reproductive traits

For reproduction, we measured and analysed the following traits: clutch size (number of eggs per clutch), inter-clutch interval (number of days between two successive clutches), egg size (diameter) and proportion of sterile eggs for the clutches for which these two later traits have been measured.

### Clutch size

The individual age trajectories of cumulative fecundity and clutch size are plotted in Figure 4. The mean clutch size trajectories of the four groups of Collembola (two clades * two food treatments) have been estimated using generalized additive models (*gam*) fitted for each of these four groups while imposing the same dimension of the basis used to represent smooth terms (k=5, Figure 5). The same value of k was used in the different models to ensure that the predicted smoothed effects of age were comparable among groups of individuals. A value of 5 was found to be sufficiently high to capture long-term non-linear effects of age of the trait's trajectories. In a second analysis, we studied the individual clutch size age trajectories by fitting a *gam* (with a k=5 as well) for each individual that died from a natural cause and that laid five clutches or more. From these fits we estimated the onset of reproductive senescence as the age at which the predictions from the *gam* reached its maximum value (Figure S5). We considered these ages as estimates of 'onset of reproductive senescence' (Figure S6), and used them to build up some survival-like curves for the initiation of reproductive senescence (Figure S7) and, as for the survival analysis detailed above, we estimated hazard functions for the four groups of Collembola to illustrate how the risk of starting clutch size decline changes with increasing age. These 'reproductive hazard functions' have been plotted alongside those of death rates to facilitate visual comparison of the timing and rate of the actuarial and reproductive senescence (Figure 3, dotted lines). We also used the individual fecundity trajectories to estimate the 'rate of reproductive senescence' as the mean slope of the decline in clutch size after the onset of reproductive senescence. We then used these estimates to look for differences in the rate of reproductive senescence among clones, clades and treatments (Figure S8). Note that what we call here for simplicity 'reproductive senescence' is in fact a *fecundity* senescence since it ignores the other dimensions of reproduction analysed below.

### Inter-clutch intervals

Reproductive senescence can affect not only the fecundity (clutch size) but also the rate at which a female reproduces. To study this aspect, we analysed if and how the time between two consecutive clutches varies with age (Figure 6). As for clutch size, we unveil the effects of age on inter-clutch intervals using generalised additive model (Table 2, Model 4). We also used a generalized linear

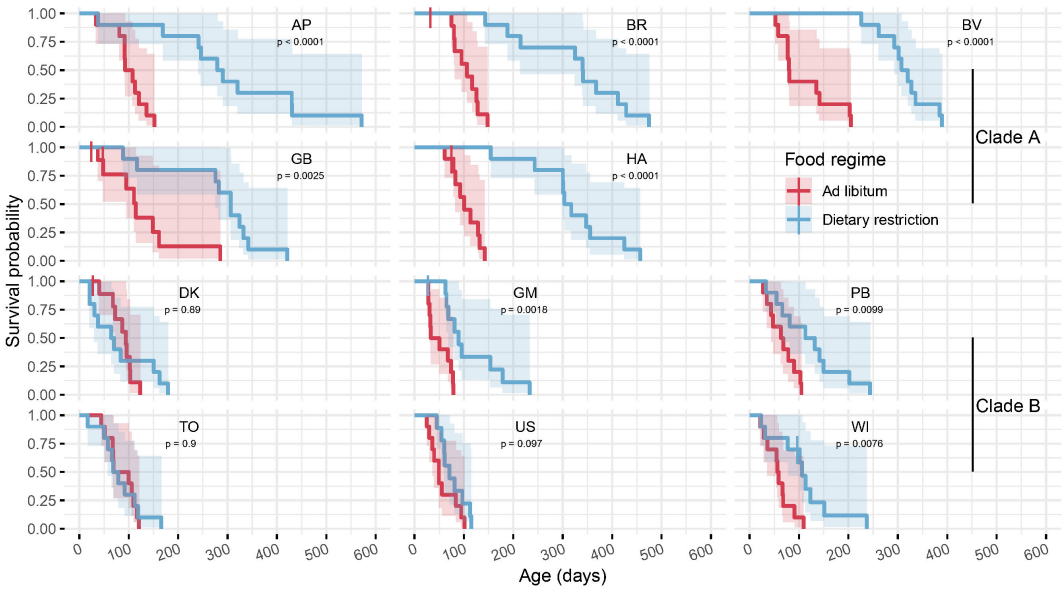


FIGURE 1
Survival curves and their associated 95% confidence intervals are plotted for each clone in the two food regimes grouped by clades (first two rows for clade A, rows 3 and 4 for clade B, 10 individuals per group of clone and food regime). For each clone, differences between food regimes are assessed with log-rank tests (printed p-values). While every clone from clade A significantly extend their lifespan when dietary restricted, the difference between the two food regimes was less pronounced or even not present in the clones from clade B.

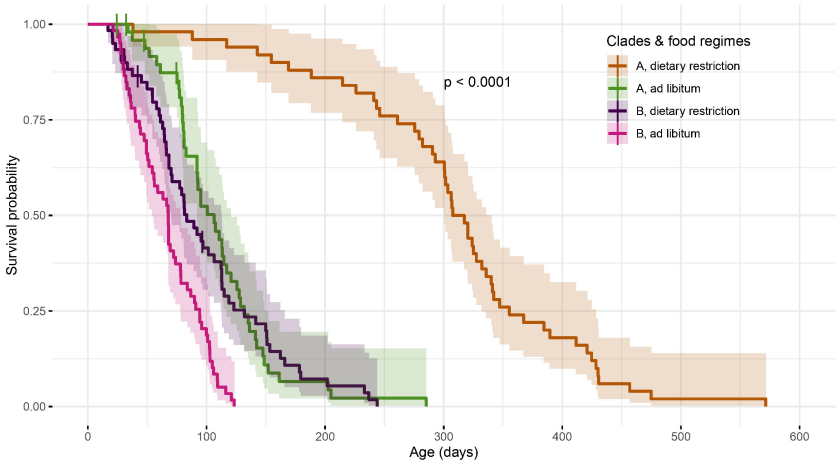


FIGURE 2
Survival curves and their associated 95% confidence intervals are plotted for the two clades and two food regimes with the p-value from the global log-rank test. Specific contrasts show that the survival curves significantly vary between the two food regimes for clade A ($\chi^2_1$ = 90, p<0.001) and also, to a lesser extent, for clade B ($\chi^2_1$ = 17, p<0.001). 100 individuals have been followed-up for each food regime for clade A and 120 collembola for clade B.

mixed model with clone and individuals as random factor to estimate fortnights and monthly estimates and their associated 95% confidence intervals (Figure 7).

### Egg size and proportion of sterile eggs

The smoothed effects of age on egg size (diameter) were plotted for each clone (Figure 8) and compared among clades and food regimes using *gam* fits (Figure 9) and linear mixed models (Table 3). As for interclutch intervals, we used a generalized mixed linear model (Gaussian) that took into account the structure of the dataset (eggs nested with clutches, nested within individuals) to estimate the mean egg diameter (and 95% confidence intervals) by fortnight periods in the four groups of clade*food regime (Figure 9). Similarly, we used generalized linear mixed models (binomial) to estimate the mean proportions of sterile eggs by fortnight periods in the four groups of individuals. For both traits, these models were fitted to the four groups of individuals only, because these traits were measured solely on a subset of the clutches which was not large enough to enable relevant comparison among clones (Figure 10).

# Results

## Lifespan and survival rates

Lifespan ranged from two weeks (17 days) to a maximum of one and a half year (571 days, Figure 1). We observed a high genetic variability of lifespan among the clones (Figure 1) and of its plastic response to food provisioning (statistically significant clone*food regime interaction, $\chi^2_{10}$ = 43.4, p<0.001, Table 1, Model 1): when grown under ad libitum food conditions, median lifespan varied from 42 days (GM, clade B) to 115 days (GB, clade A) and under caloric restriction, from 67 (DK, B) to 341 days (BR, A). For clade A, the five clones responded similarly to caloric restriction by a remarkable increase of the mean lifespan (x 2.82 on average among the clones, Figure 1 and Table 1). For clade B, the effect of caloric restriction was less pronounced (x 1.96 on average, nonetheless, for the clones GM, PB, WI) or even negligible for three clones (DK, TO, US, Figure 1 and Table 1).

By grouping the clones within each clade, we found that (1) in both environments, the clade A had on average a lower mortality and thus a higher longevity than clade B (Figures 2, 3 and Table 1) and (2) that on average, the magnitude of the positive effect of food deprivation on lifespan varied dramatically among the two clades: the amplitude of the right shift of the survival curves under dietary restriction is remarkably singular for clade A whose mean longevity almost tripled under dietary restriction (Figure 2). This genetic*environment interaction (Table 1, Model 2, $\chi^2_1$ = 25.6, p<0.001) is also reflected in the shapes of the mortality trajectories (Figure 3, solid lines): based on the visual comparison of these trajectories and of the overlap of their 95% confidence bands (see Figure S4), under each food regime, individuals from clade B suffered on average from a higher initial basal mortality rate (before ~50 days), an earlier onset of senescence and a tend to suffer from a steeper increase of mortality with advancing age. Clade A is also remarkable by its low mortality and almost negligible actuarial senescence under dietary restriction. Under ad libitum food regime, the onset of senescence – which is not easy to quantitatively determine - seems also delayed compared to clade B and the increase in mortality rate was not only slow, but also visually seemed to reach a mortality plateau (green trajectory, Figures 3, S4).

## Clutch size

Clutch size ranged from a minimum of one egg to a maximum of 178 eggs per clutch with a mean of 39 and a median of 26 eggs (Figure 4A) and the maximum number of eggs laid during a whole lifespan reached 1298 (Figure 4B). Not surprisingly, we found a

TABLE 1 Survival.

| Characteristic | Lifespan (days) | HR[1] | 95% CI[2] | p-value | Global p-value |
|---|---|---|---|---|---|
| Model 1: Clones*food regimes | | | | | |
| **Clones** | | | | | <0.001 |
| *AP (+)* | 100 | 1.00 | — | | |
| *BR* | 106 | 0.96 | 0.39, 2.36 | >0.9 | |
| *BV* | 80 | 0.76 | 0.31, 1.84 | 0.5 | |
| *GB* | 115 | 0.47 | 0.18, 1.24 | 0.13 | |
| *HA* | 101 | 0.97 | 0.40, 2.40 | >0.9 | |
| *DK* | 94 | 1.56 | 0.63, 3.86 | 0.3 | |
| *GM* | 42 | 5.69 | 2.30, 14.1 | <0.001 | |
| *PB* | 66 | 2.86 | 1.18, 6.96 | 0.021 | |
| *TO* | 85 | 1.57 | 0.65, 3.78 | 0.3 | |
| *US* | 49 | 3.79 | 1.55, 9.25 | 0.003 | |
| *WI* | 57 | 3.67 | 1.50, 8.95 | 0.004 | |
| **Food regimes** | | | | | <0.001 |
| + | | 1.00 | — | | |
| *- (AP)* | 285 | 0.03 | 0.01, 0.10 | <0.001 | |
| **Clones * Food regimes** | | | | | <0.001 |
| *BR * -* | 341 | 1.02 | 0.28, 3.68 | >0.9 | |
| *BV * -* | 314 | 2.11 | 0.57, 7.81 | 0.3 | |
| *GB * -* | 307 | 3.93 | 1.01, 15.3 | 0.048 | |
| *HA * -* | 310 | 1.23 | 0.34, 4.48 | 0.7 | |
| *DK * -* | 67 | 20.9 | 5.00, 87.5 | <0.001 | |
| *GM * -* | 89 | 3.85 | 0.92, 16.1 | 0.065 | |
| *PB * -* | 122 | 6.25 | 1.54, 25.3 | 0.010 | |
| *TO * -* | 74 | 27.9 | 6.76, 115 | <0.001 | |
| *US * -* | 71 | 16.2 | 3.83, 68.3 | <0.001 | |
| *WI * -* | 106 | 6.02 | 1.45, 25.0 | 0.013 | |
| Model 2: Clades* Food regimes | | | | | |
| Clades | | | | | <0.001 |
| *A (+)* | 106 days | 1.00 | — | | |
| *B (+)* | 68 | 2.96 | 1.97, 4.47 | | |
| Food regimes | | | | | <0.001 |
| + | | 1.00 | — | | |
| *- (A)* | 312 | 0.06 | 0.03, 0.12 | | |
| Clades * Food regimes | | | | | <0.001 |
| *B * -* | 83 | 5.96 | 2.89, 12.3 | | |

[1]HR, Hazard Ratio; [2]CI, Confidence Interval.

This table summarizes the output of a Cox proportional hazard model with clones, food regimes ("+" for ad libitum food and "-" for dietary restriction) and interaction between clones and food regimes as covariates. The reported hazard ratio (HR) and associated p-values measure if and how the risk of dying differs from the reference (clone AP). A value significantly greater than 1 suggests an increased risk of dying compared to the reference, and a smaller risk is revealed by a HR<1. Fully fed GM have a media lifespan of 42 days and endure on average a 5.7 higher risk of dying compared to fully fed AP collembola. Dietary restricted TO suffer from a 28 higher hazard of death compared to dietary restricted AP. And the hazard rate of fully fed AP is around thirty times higher than for dietary restricted AP (1/0.03).

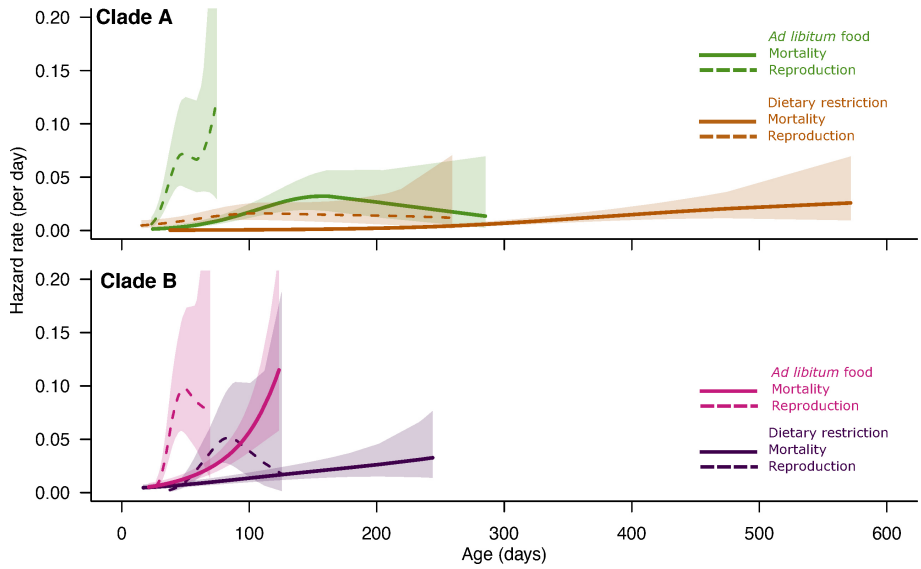


FIGURE 3
The estimated age trajectories and their associated 95% confidence intervals of daily death rate (solid lines) and daily risk of starting a reproductive decline (clutch size, dotted lines) for the four groups of Collembola (two clades under the two food regimes).

strong effect of food regime on clutch size in every clone (Figure 4A) and clade (Figure 5). Over the whole lifespan, the mean clutch size under ad libitum food regime was 73 eggs, and 72% of the laid clutches were larger than 50 eggs. As expected, under dietary restricted conditions, the clutches remained much smaller (20 eggs on average, 95% of the clutches having fewer than 50 eggs). Both age and food regime influenced the clutch size: the first clutch laid under dietary restriction was on average half the size (11 eggs) of the ones laid in ad libitum food conditions (22 eggs). This strong effect of food regime on fecundity amplified as the Collembola became older (Figure 5) and larger (Figure S1).

Under ad libitum food regime, the mean fecundity progressively increased up to reaching a maximum after about 40 or 50 days (Figure 5). Following this productive period, the fecundity of the Collembola still alive begins to decline then eventually becomes null, a certain number of springtails managing to survive long after laying their last clutch (horizontal ends of cumulative fecundity trajectories visible on Figure 4B and analysed in detail in a previous paper (Tully and Lambert, 2011)). It a nutshell, we found that most clutch size age trajectories were bell-shaped: an initial increase, followed by a narrow maximum and a progressive decline. The mean trajectories adjusted for the four groups of Collembola (two clades * two food regimes, Figure 5) brings out the higher fecundity (and higher lifetime fecundity) of clade B compared to clade A, especially under the ad libitum food regime (Table 2, Model 3). Figure 5 shows not only the positive effect of ad libitum food on fecundity in both clades but also the delayed and slower fecundity decline under dietary restricted conditions. One can also remark that, as for mortality, the negative effect of age was barely noticeable under dietary restricted regime for clade A (negligible senescence).

To better compare the age distribution of the onset of clutch size senescence with the shape of the mortality age trajectories, we produced some "mortality like trajectories" of the daily risk of starting a reproductive decline using the individual's estimates of the onset of clutch size senescence (Figures S5–S7). We found that the shape of the "age trajectories of the risk of starting a reproductive decline" varied among clades and food regimes in a manner that echoed the observed variations among the mortality trajectories (Figure 3, dotted versus solid lines). In clade A, under dietary restricted conditions, as for mortality, the risk of starting a reproductive decline remained low with a negligible effect of age. For the three other groups of Collembola, the hazard rate for clutch size senescence started to increase earlier and at a higher rate than for mortality. Interestingly, the relative order between the different trajectories were maintained: in both clades, the hazard rates started to increase with age earlier and faster under ad libitum conditions for both mortality and fecundity.

Once reproductive senescence had started, the clutch sizes declined on average at a higher rate in fully fed springtails than in dietary restricted ones (Figure S8).

## Inter-clutch intervals

The intervals between successive clutches ranged from at least half a week (3.5 days) to over five months (172 days for an old GB female). Well-fed females had on average a higher spawning frequency than females on diet, with a median value of about a week in high food conditions and 12 days in low food conditions. This effect of food regime is visible for each clone especially during the first two months (Figure 6). The *gam* revealed that, for each lineage and each food regime, the egg-laying frequency tended to slow down with age (Figure 7 and Table 2, Model 4). The pace of this slowdown appears to be somehow irregular: during the first part of their reproductive life, most adults managed to maintain a more or less regular or barely slowing spawning rate, but after about

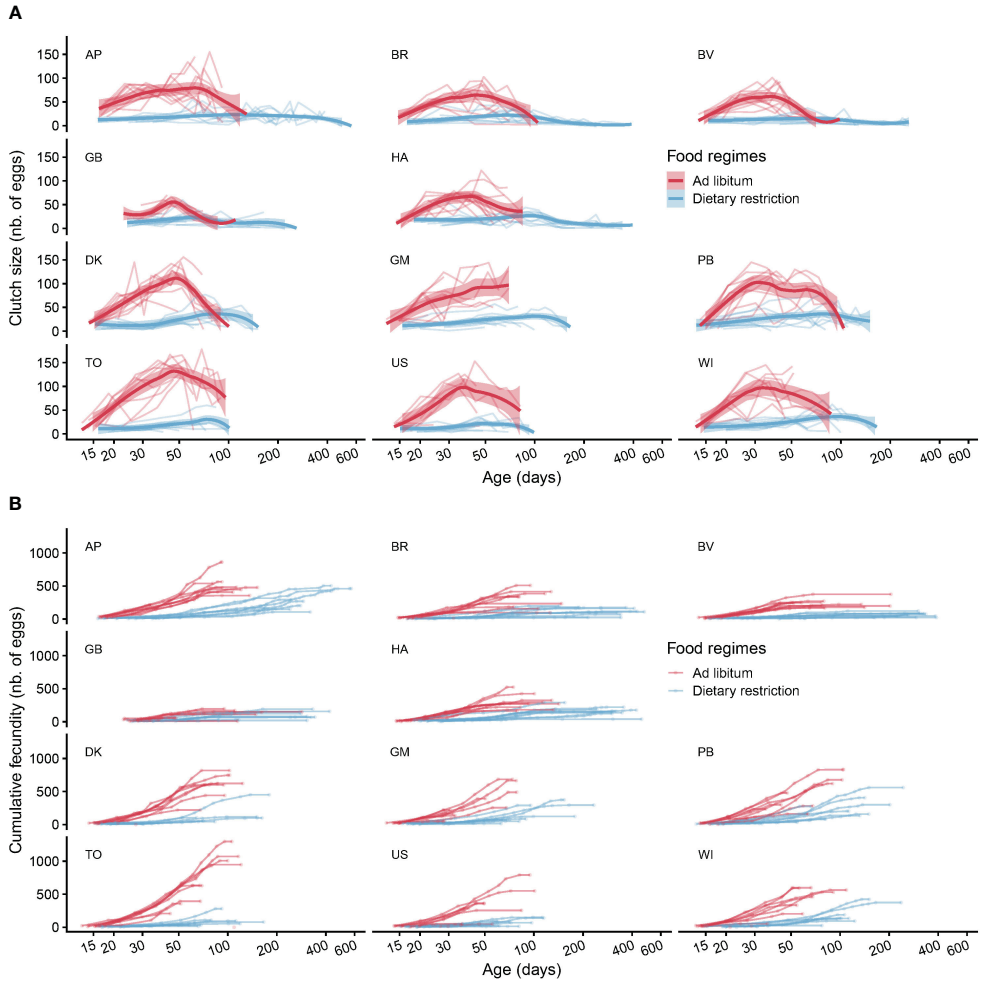


FIGURE 4
Clutch size age trajectories and cumulative fecundity. The panel **(A)** displays the individual clutch size age trajectories for each clone and food regimes together with smoothed curves to underline the mean effects of age on clutch size. The panel **(B)** represents the individual trajectories of cumulative fecundity. Each circle on the figure represents an egg-laying event, except for the last circles at the end of each trajectory, which represents the death of the individuals. In each panel the clones from the first two rows belong to clade A and the clones from the rows 3 and 4 belong to clade B.

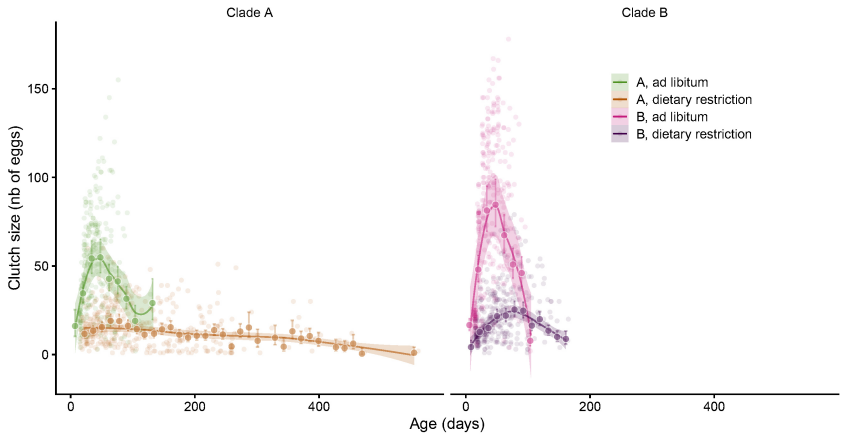


FIGURE 5
Mean clutch size age trajectories for each clade in the two food regimes. The small pale circles represent the raw data. The large circles and associated 95% confidence intervals are the fortnight's means estimated from a linear mixed model with individuals nested within clones as random factors. Smoothed trajectories and their 95% confidence bands estimated from four independent generalized additive models have been added to underline the non-linear shapes of the mean fecundity trajectories.

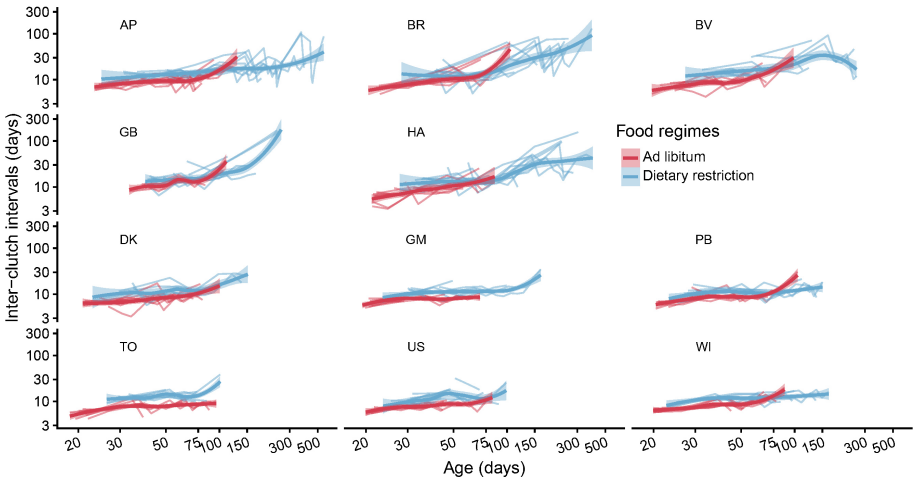


FIGURE 6
The age trajectories of the inter-clutch intervals (number of days between two consecutive events of egg laying) are plotted for each individual (thin lines, individual trajectories). The thick curves represent the mean smoothed trajectories for each clone and food regime. Note that, to better reveal the details, the data have been plotted on logarithmic scales both for the x- and y-axis. This graph reveals that on average the beginning of reproduction is delayed under dietary restriction and the pace of reproduction is also reduced when food is scarce. This figure also shows the progressive increase in the inter-clutch intervals as the springtails become older.

75 days for well-fed individuals the mean interval between successive clutches started to increase markedly (breaks in slope visible in the *gam* of Figure 7). Such discontinuity was less visible for dietary restricted individuals whose spawning rate decreases more gradually with age.

As time goes on, the speed of this increase became typically higher in well-fed individuals such that for several clones and for the two clades on average, after a certain age (~80-100 days), the rate of clutch production of the well-fed individuals became lower than that of their same-age dietary restricted congenerics (cf. intersecting smoothed curves in Figures 6, 7). The mean effect of age on inter-clutch intervals appeared to be roughly similar between the two clades (Figure 7) despite some marked differences between some clonal lineages (Figure 6).

## Egg size

Egg diameter was on average 146 µm and ranged between 2 and 189 µm. Despite some marked variation in egg size between and

TABLE 2 Clutch size and inter-clutch intervals.

| Characteristic | Model 3: clutch size | | | | Model 4: inter-clutch intervals | | | |
|---|---|---|---|---|---|---|---|---|
| | Beta | 95% CI | t value | p-value | Beta | 95% CI | t value | p-value |
| Parametric part of the model | | | | | | | | |
| A, + (reference) | 9.2 | 4.9, 13.6 | 4.15 | <0.001 | 2.6 | 2.48, 2.68 | 50.5 | |
| A, - | -6.6 | -11, -2.2 | -2.96 | 0.003 | 0.13 | 0.01, 0.23 | 2.2 | 0.027 |
| B, + | 0.03 | -0.41, 0.48 | 0.15 | 0.9 | -0.20 | -0.28, -0.12 | -4.4 | <0.001 |
| B, - | -7.0 | -12, -2.1 | -2.8 | 0.005 | -0.07 | -0.17, 0.04 | -1.16 | 0.25 |
| **Smoothed effects of age on** | Edf | | | p-value | | | Edf | p-value |
| A, + (reference): | 7.2 | | | <0.001 | | | 6.8 | <0.001 |
| A, - | 7.1 | | | <0.001 | | | 2.7 | <0.001 |
| B, + | 2.8 | | | <0.001 | | | 1.0 | 0.002 |
| B, - | 5.5 | | | <0.001 | | | 3.1 | <0.001 |

Beta, parameters estimates; CI, confidence intervals; EDF, Effective degree of freedom.
Results from generalized additive mixed models (*gamm4* function from the *gamm4* library) used to analyse the effects of clades and food regimes ("+" for ad libitum food and "-" for dietary restriction) on (1) clutch size (model 3, Poisson family) and (2) inter-clutch intervals (model 4, intervals in days, and log10 transformed) while using a separate smoother for each of the four groups of Collembola to take into account the effect of age on each group of clade and food regime. A variable that identifies each individual was included in the random part of the models. Including "clone" as a random factor was not possible due to convergence issues. The tests refer to pair-wise comparisons of the parametric parts of the model and of the smoothed effect of age (in days) to a reference level, here clade A fed ad libitum (+). Age was log-transformed for the model on the inter-clutch interval to correspond to the Figure 6.

within clutches (Figure 8), and contrary to the other studied traits, the visual inspection of the smoothed age trajectories among clades (Figure 9) indicates that Collembola managed to maintain a relatively constant egg size during their whole lifespan even if a closer look suggests that Collembola age can have some marginal effects on egg size under some conditions.

Given the structure of the data (eggs nested within clutches, clutches within individuals and individuals within clones) and the relatively flat or linear effect of age revealed by the *gam* models, we used linear mixed models to study if age had indeed a detectable negative effect on egg size. We found evidence of interactions between age and clade (p=0.026), and between age and food regimes (p=0.004, see Table 3, Model 5 & Model 6). The study of sub-models (Table 3, Models 7-11) revealed that the mean size of eggs laid by well-fed springtails was slightly larger in clade B (p<0.001) but did not vary with increasing age (p=0.13, negligible senescence, Model 8). It is only in the long-lived dietary restricted clade A that we found a decline of egg size with increasing age (p<0.001, Model 10 & Figure 9). This effect, especially due to some very old individuals, was, however, quantitatively modest: the mean diameter of the eggs produced by Collembola older than 180 days was indeed only 6% smaller than those laid by adults younger than 80 days old (Figure 9).

## Proportion of sterile eggs

On average, over the whole lifespan, about 6% of the eggs were sterile. This proportion varied among clutches and ranged between 0% (for 40% of the clutches) and up to 100% for a few of them. Clade B produced on average 2.5% of sterile eggs while this proportion was seven times higher in clade A on average (17.5%). The proportion of sterile eggs changed with age but differently depending on the clade and food regime (significant age*food*clade interaction, p=0.002, Table S1). For clade A, the proportion of sterile eggs increased with age but at a slower rate in the dietary restriction regime. For Clade B, the effect of age also varied among treatments: old Collembola produced on average more sterile eggs when fed ad libitum while the proportion of sterile eggs dropped to almost zero in old dietary restricted springtails (Figure 10, Table S2).

# Discussion

## Lifespan and actuarial senescence are genetically and phenotypically variable

Using our unique longitudinal dataset, we were first able to show that different strains of *F. candida* can have contrasting lifespans, as has been shown in wild populations of various species like the fruit fly *Drosophila melanogaster* (Grandison et al., 2009), the rotifer *Brachionus* (Gribble et al., 2018), or the nematod *Caenorhabditis elegans* (Chen et al., 2001). We also found that Collembola can suffer from actuarial senescence and that the onset and the rate of senescence were both, at the same time, phenotypically plastic and genetically variable. This echoes previous research where we found that, in protected laboratory conditions (16°C, constant density), mortality rate of *F. candida* can increase exponentially with age and that different components of the shape of the mortality trajectories can vary among genetically distinct lineages (Mallard et al., 2015). Even if the conditions varied between the two experiments, it is interesting to notice that the clonal lineage that was able to delay remarkably the onset of actuarial senescence in this former publication belongs to clade A which is the one that also had here a delayed onset and slower ageing rate. Thus, this shows that the qualitative difference observed between the two clades can remain in different environmental and demographic contexts.

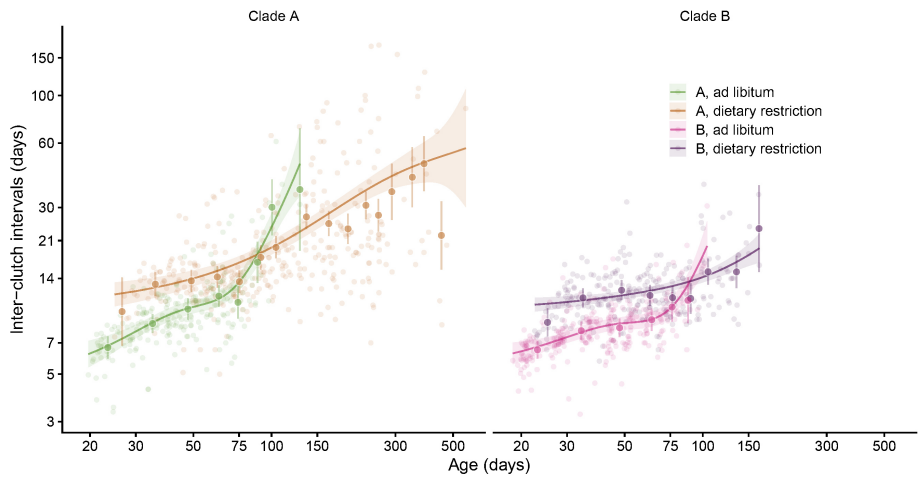


FIGURE 7
This graph displays the mean smoothed age trajectories (estimated with gams) for each combination of clade and food regimes over the raw measurements (pale circles). We also added the means and 95%CI of the inter-clutch intervals over fortnights (between 0 and 100 days), and over one month (between 100 and 300 days) or two months for measurements made after 300 days. We used a generalized mixed model with individuals and clones as random effects to estimate these means (large circle). Note that to increase the readability, we have used logarithmic scales for the x- and y-axis.

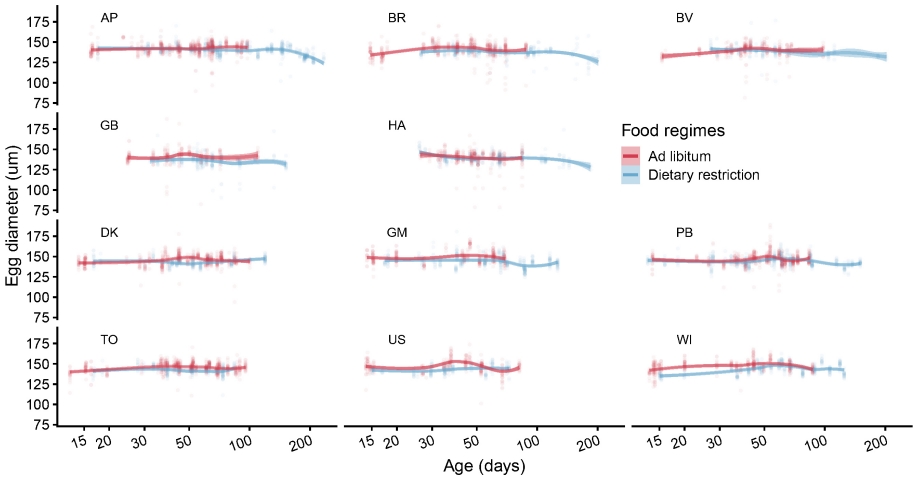


FIGURE 8
Egg size (diameter, mm) as a function of springtail's age. This figure displays the row measurements made on various clutches produced by different Collembola raised under the two food regimes (pale circles). The data are sorted by clade and clones within each clade. A smoothed curve is adjusted to each combination of clone and food regime.

## The effects of dietary restriction on lifespan, mortality and reproductive trajectories are remarkable

As expected from a very large number of empirical studies (Kirkwood and Shanley, 2005; Piper et al., 2011), including this Collembola (Tully and Lambert, 2011), we found that in dietary restricted conditions, our springtails managed to extend their longevity. This can be achieved either by reducing the short-term mortality rate (Mair et al., 2003; Mair et al., 2005), by delaying the onset of ageing or of associated health disorders (Mattison et al., 2012) or by slowing the rate of ageing (Merry, 2005).

Contrary to what has been observed in *Drosophila melanogaster* (Partridge et al., 2005) or spider mites for instance (Li and Zhang, 2021), where dietary restriction lowers the risk of death but affects only marginally the rate of ageing itself, dietary restricted Collembola achieved lifespan extension by maintaining a low basal adult mortality rate and by setting back the onset of actuarial senescence and slowing its rate. This plasticity, especially remarkable in one clade (A), enabled the springtails not only to double or almost triple their life expectancy but also to change the qualitative pattern of senescence: a change in diet can trigger a shift from strong negative actuarial senescence to negligible senescence. Such dramatic plastic changes resemble the ones that we described in the meadow vipers where, depending on the microhabitats, ageing vipers could either suffer from severe decline of survival or benefit from negligible senescence (Tully et al., 2020). These similarities among such distant species imply that this level of age-trajectory plasticity may be more common than one could imagine. More empirical studies in the wild and in the laboratory

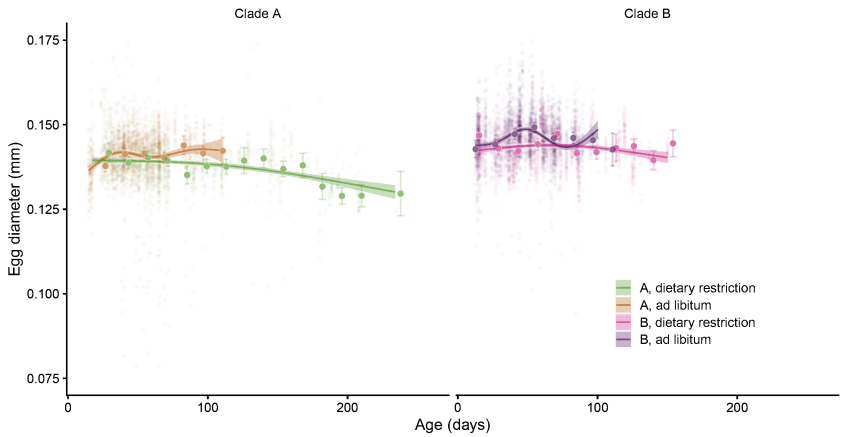


FIGURE 9
Egg size (diameter, mm) as a function of age. This figure represents the trend estimated for the two clades in the two food regimes and the mean (and 95% confidence interval) egg size estimated per fortnight for each of the four groups of individuals. These estimates come from a linear mixed model with clutches, individuals and clones as nested random factors.

TABLE 3 Egg size.

| | Model 5: Full model | | Model 6: Simplified model | | Model 7: Ad lib only, full model | | Model 8: Ad lib, simplified | | Model 9: DR only, full | | Model 10: DR, clade A | | Model 11: DR, clade B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Predictors | Beta | P | Beta | P | Beta | p | Beta | P | Beta | P | Beta | P | Beta | P |
| **A, +** | 139.8* | <0.001 | 140.7* | <0.001 | 139.80* | <0.001 | 139.92* | <0.001 | | | | | | |
| **Age (days)** | 0.02 | 0.325 | 0.00 | 0.930 | 0.02 | 0.268 | 0.02 | 0.132 | -0.05 | <0.001 | -0.05 | <0.001 | -0.00 | 0.886 |
| **B, +** | 5.87 | <0.001 | 4.36 | <0.001 | 5.88 | <0.001 | 5.67 | <0.001 | 1.83 | 0.154 | | | | |
| **A, -** | 2.2 | 0.091 | 1.08 | 0.293 | | | | | 142.0* | <0.001 | 142.1* | <0.001 | | |
| **B, -** | -4.06 | 0.022 | -1.88 | 0.031 | | | | | | | | | 143.8* | <0.001 |
| **Age × clade [B]** | -0.00 | 0.927 | 0.03 | 0.026 | -0.00 | 0.868 | | | 0.04 | 0.006 | | | | |
| **Age × food [-]** | -0.07 | 0.002 | -0.04 | 0.004 | | | | | | | | | | |
| **Age × Clade [B] × food [-]** | 0.04 | 0.158 | | | | | | | | | | | | |
| **Numbers** | 11 clones, 189 springtails, 583 clutches, 9834 eggs | | | | 11, 94, 300, 6013 | | | | 11, 95, 283, 3821 | | 5, 47, 155, 1756 | | 6, 48, 128, 2065 | |
| **Marginal $R^2$/ Conditional $R^2$** | 0.117/0.309 | | 0.119/0.311 | | 0.080/0.259 | | 0.081/0.259 | | 0.130/0.373 | | 0.081/0.278 | | 0.000/0.338 | |

Results from mixed models (*lmer* from *lme4* library) analysing the effect of clades (A/B), food regime (ad libitum "+" versus dietary restriction "-") and age on the egg diameter (µm). Variables identifying clutches, individuals and clones were included as nested random effects. We present the full model which reveals several significant interactions and several sub-models to explore the effects responsible for the interactions. In a nutshell, we found a significant (negative) effect of age only in the dietary restricted clade A individuals (Model 10). Model 5 is the full model with the triple interaction. Model 6 is the simplified model with the second order interactions only. Model 7 and its simplified version Model 8 - the sub-models in the ad libitum food environment only - show that individuals from clade A lay on average smaller eggs than clade B and for neither clade does egg size change with age. Models 9, 10 and 11 are the sub-models on the dietary restricted environment only. In this environment, age has a significant negative effect on the egg size on clade A only. Asterisks indicate the parameters estimates (beta) that correspond to the models' intercepts. For Model 9 for instance, the predicted mean egg diameter for dietary restricted Clade A is 142 µm which is not significantly different from clade B (142 + 1.83 µm, P=10.15). This diameter decreases on average by 0.05 µm/day as the collembola from clade A become older while this decrease is negligeable for clade B (-0.05 + 0.04).

are required to assess how common are these plastic changes in the shape of the mortality age trajectory. If found to be widespread, this plasticity may call into question the generality of conclusions drawn from comparative studies that do not consider the possibility of a crucial role of environmental conditions on the shape of the trajectories studied.

This plasticity could also be linked to two common properties of these species, namely continuous growth and a fertility that increases with adult size, given that these features are linked to the evolution of negligible senescence (Vaupel et al., 2004; Baudisch, 2008; Baudisch and Vaupel, 2010). In other words, it can be especially advantageous to be able to survive long periods of

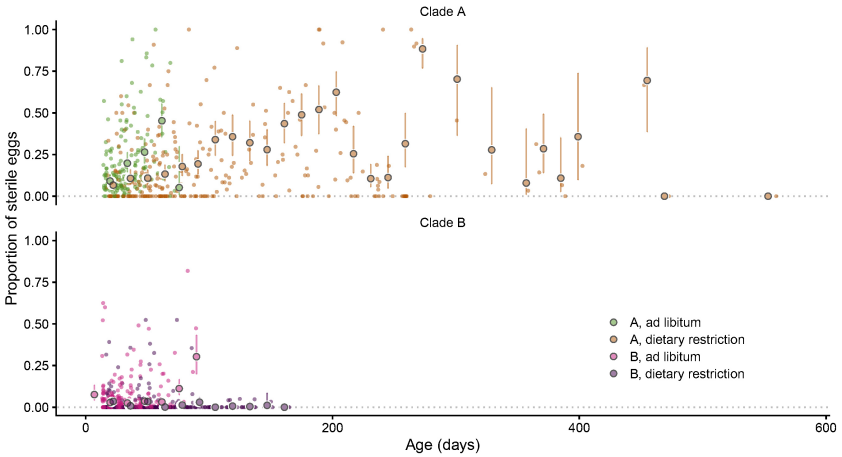


FIGURE 10
The proportion of sterile eggs has been measured for 404 clutches (small circles). The mean and 95% CI of the proportion of sterile eggs has been estimated every fortnight for the four groups of Collembola (large filled circles and error bars) with a binomial linear mixed model with clutches, individuals and clones as randoms effects.

fasting if you are able, as an adult, to resume growth then benefit from an especially high fecundity once better days have come. It would be worth exploring the Collembola's responses after a return to an ad libitum diet succeeding a period of fasting to better assess if dietary restricted induced lifespan extension coupled with slackening reproduction is indeed an adaptive response (Sultanova et al., 2021).

Regarding reproduction, nothing unexpected in the fact that undernourished Collembola lay smaller clutches and less often. The novelty of our work is rather to quantify effect of dietary restriction on the timing and pace of reproductive decline on several traits simultaneously that we discuss in more details below.

## Collembola suffer from reproductive senescence in multiple ways

Our study offers a unique opportunity to describe and compare how chronological age alters various dimensions of reproduction. Apart from egg size, all reproductive traits declined markedly with increasing age: ageing springtails on the wane lay smaller clutches, less often, and with on average a larger proportion of sterile eggs. This shows that senescence can touch nearly all the dimensions of reproduction, with non-linear effects and some abrupt accelerations.

We also found notable differences between clones and clades for all the traits analysed, which shows that several parameters of reproductive ageing still possess the potential to evolve. The comparison of the two clades also discloses a potential link between reproduction and survival: the clade that suffers from an earlier and more intense actuarial senescence is also the one with the highest reproductive potential (as also discussed in (Tully and Ferrière, 2008)) which supports the idea that a trade-off between somatic maintenance and reproduction may be at the heart of the ageing process, following the logic of the disposable soma theory (Kirkwood and Austad, 2000; Kirkwood, 2002).

We also found an odd difference between the clades, namely in the proportion of sterile eggs. The more fertile clade was the one with the lowest level of sterile eggs even at old ages. Given the implication of the endosymbiont *Wolbachia* in this species' reproduction and parthenogenesis (Frati et al., 2004), one could speculate that part of the difference observed between the clones could be linked to differences in *Wolbachia*'s loads, given that it has been shown that *Wolbachia* are essential for embryonic development and egg hatching in this species (Pike and Kingcombe, 2009; Timmermans and Ellers, 2009; Hafer and Pike, 2010). Our results also encourage exploring the potential effects of ageing on *Wolbachia*'s load and, in turn, the potential effects of *Wolbachia*'s load on the age trajectories, as is the case in *Drosophila melanogaster*, where the presence of this endosymbiont increases lifespan by delaying the onset of actuarial senescence (Alexandrov et al., 2007).

As expected, we found that dietary restriction reduced the reproductive output: poorly fed Collembola lay smaller clutches and at a slower pace. We also found that dietary restriction had, as for survival, a protective effect on the reproductive ageing trajectory: the onset of reproductive senescence was delayed and the reproductive decline when it has started was more gradual. Such protective effect has been observed in a mite (Li and Zhang, 2021) where dietary restriction delayed the onset of reproductive decline. As for survival, this protective effect was large enough in one clade - clade A - to get rid of almost any negative effect of age on fecundity. The slow rate of decline in fertility in starving Collembola (Figure S8) could also be determined by a protective effect of dietary restriction, but one cannot rule out the fact that this could also be a simple statistical effect: the fall being all the faster the higher you start.

## Actuarial and reproductive senescence are asynchronous but not unrelated

The joint study of reproduction and survival enabled us to compare the timing of senescence of various traits. We provide another empirical evidence of the asynchrony of senescence (Cayuela et al., 2020b): the onset of senescence varied between traits and among genetic lineages and environmental backgrounds.

Behind this diversity, we found some regularity in the timing of senescence among different traits. Negligible mortality senescence was associated with a marginal decline in fecundity, a low and roughly constant risk of starting a reproductive decline and a slow and progressive slowdown of the rhythm of reproduction, with no jolt. In this case the pace of ageing was faint or barely noticeable for every trait analysed. We did not observe a combination of marked actuarial senescence with negligible reproductive senescence as has been observed in snakes for instance (Cayuela et al., 2020b; Tully et al., 2020). Whenever senescence was tangible, we found that clutch size senescence was followed closely by a marked increase in the inter-clutch intervals and the senescence of these two components of reproduction precede the observed rises in mortality rates. It is as if, after reaching a reproductive peak, the Collembola became reproductively exhausted but were still able to postpone their death of old age until they could capitalize on their whole reproductive lifespan. This is indeed coherent with our previous finding of a remarkably long post-reproductive lifespan (Tully and Lambert, 2011), and the selective forces that may have acted to select for such a gap between the end of reproductive life and the end of somatic life can be called upon to explain the systematic shift observed between reproductive and actuarial senescence. This may also explain similar observations in other species (Reznick et al., 2006; Auld, 2018; Nielsen et al., 2021).

Contrary to all the other traits examined, we found negligible senescence on egg size in the two clades and two food regimes. Similarly, no negative effect of old age was found on mean egg size in quails (Vedder et al., 2022), Turtles (Congdon et al., 2003), bugs (Kasule, 1991) or on neonate size in the meadow viper (Tully et al., 2020) while a negative effect of age on egg volume has been reported in a long-lived sea bird (Beamonte-Barrientos et al., 2010). This later effect was, however, quantitatively marginal and inconsequential on hatching success. A recent study on polar bears also found no senescence of the maternal effects: on the contrary, old females produced cubs whose survival tended to be higher from that of congeners born form younger and fitter - but probably also less experienced – females (Naciri et al., 2022).

These various evidences support the idea that some components of reproduction are less susceptible to decline with age than others. Egg size is highly variable in this species (Stam et al., 1996) and we have shown that juveniles hatched from large eggs survive better than

those from small eggs in conditions where food is scarce (Tully and Ferrière, 2008). Given its fundamental role in determining the offspring chance to survive to adulthood, egg size - or size at birth - is under severe selective pressure, especially if there is a certain threshold size below which development or survival becomes abruptly impossible. Such strong selection could explain why egg size, in our Collembola and in the above-mentioned empirical examples, appears to be so canalized, i.e. phenotypically consistent, in face of increasing mothers' age, despite some potential plasticity or flexibility in egg size (Liefting and Ellers, 2008; Tully and Ferrière, 2008; Liefting et al., 2010). Natural selection has likely favoured individuals who manage to maintain egg size above a critical lethal threshold even at advanced ages, when other components of reproduction whose lessening is not as critical for female fitness have already progressively declined with age. Such difference in the shape of the functions linking a trait's value to an individual's fitness could help understand the evolutionary origin of the observed diversity and asynchrony in senescence's pathways.

We still have to reconcile our present finding with the previous observation that the Collembola reproductive decline was subsequent to the onset of actuarial senescence (Mallard et al., 2015). In the latter case, individuals were kept and maintained at a colder temperature and by groups of 10, rather than isolated. Low temperature and intraspecific competition may have created conditions favourable for delaying the onset of reproductive ageing. Following groups of individuals prevented a close follow-up and quantification of the onset of reproductive ageing based on individual trajectories as we have done here. Conversely, Mallard et al. used many more Collembola that in this study which let them describe with a fine resolution the shape of the mortality trajectory which probably helps detect earlier onsets. This also underlines that determining the onset of senescence can be methodologically challenging, and may be biased by the quality of the data collected. More experimental and theoretical works are also required to help identify if some environmental conditions could lead to reversals in the relative order of ageing of different traits.

We would finally like to pay particular attention to the method that we used to compare the senescence of mortality and reproduction. Describing and comparing the shapes of the age trajectories of reproduction is always informative, but deriving from these trajectories an estimation of the hazard rate of becoming reproductively senescent uncovers a new telltale signature of the process of reproductive senescence. The advantage of this method is that reproductive hazard rate trajectories can be directly compared to the death rate trajectories, and they even share the same units. It could be interesting to test this novel approach on other empirical or theoretical examples to assess its usefulness in ageing research.

## Study limitations and future directions

### The advantages and limitations of individual monitoring

A strong point of this study is undoubtedly the fact that we carried out individual monitoring, which eliminates all the problems of density that can covary with age during cohort monitoring. Our individual monitoring also enabled us to detect the effects of age on the various reproductive traits in a robust and unbiased manner. However, this is at the expense of the number of individuals monitored. For logistical reasons, we were only able to monitor 10 individuals per clone and treatment, which is low, particularly for estimating survival curves. These small numbers may be at the origin of the stochasticity observed in the patterns of survival curves in Figure 1. Inter-clone comparisons, particularly for survival, are therefore undoubtedly fragile because of this limitation. On the other hand, inter-clade comparisons, which allow several clones to be grouped together, are based on a hundred or so individuals, which makes it possible to obtain more robust results.

### Considering the effects of age-covarying body size

To stay as close as possible to the traits analysed and avoid making the analyses too complex, we ignored here the covarying change of springtail body length with age. Collembola, like snakes, start to reproduce largely before reaching their asymptotic size, and they continue to mould and grow during their whole life. Ignoring the confounding effects of body length with age, in such indeterminate growers whose fecundity is proportional to size (as can be seen in Figure S1 for our springtail), can mask some potential negative effects of age, and this could lead to the questionable conclusion that there is no senescence (Sparkman et al., 2007; Tully et al., 2020). In the present case, we found marked signs of senescence despite this potential bias, and negligible senescence when observed, was revealed largely over a period during which the Collembola stopped growing. This supports the idea that we provide conservative results, although considering the effect of size could lead to the detection of slightly earlier onsets of reproductive senescence.

### Exploring the importance of within-clade genetic diversity

We have explored the shapes of the age trajectories by grouping together clonal lineages belonging to the two phylogenetically distinct clades. This does not mean that within each clade the clonal lineages are perfectly similar. Indeed, although reduced, there is a certain level of genetic diversity within each clade. Although the number of replicates per clone is low to quantify precisely potential differences among clades, one cannot rule out the fact that part of the senescence phenotypes that we describe here could result from among-clones differences. For instance, the observed increase over time of the proportion of sterile eggs in some conditions can result either from a phenotypic change that impacts potentially all the individuals, or from an effect of selective mortality - or of reduction in fecundity - that could affect predominantly the lineages that have intrinsically a lower level of sterile eggs. More experiments, with a higher number of replicates per clonal lineages, could help ascertain what is due to genetic or to non-genetic variance.

### Exploring the links between traits and their potential modification with age

A fourth limitation is that we did not explore the potential links and correlations between the different traits examined here. Yet, the fact the different traits have been measured on isolated individuals make it feasible to search for genetic, plastic, or physiological compensations

between pairs of traits like clutch size and inter-clutch intervals, clutch size and egg size. One could also study if and how such potential physiological trade-off vary while the springtails become older. Other classical trade-offs between longevity (lifespan, onset or rate of senescence) and reproduction (reproductive output, or early reproduction) could have been studied. Finally, it could also be worth exploring if the onset and the rate of reproductive senescence are linked to each other or if these two later traits are linked to longevity for instance.

### Investigating the transgenerational consequences of senescence

Senescence can also manifest itself through long-term transgenerational effects. The offspring produced by ageing individuals can have a lower fitness than offspring born form younger parents. In the woodlice *Armadillidium vulgare* for instance, an indeterminate grower and iteroparous invertebrate as Collembola, old females produce larger clutches with larger eggs than young adults, but the offspring that hatches form these eggs had a lower survival rate than those issued from young adults (Depeux et al., 2020). This shows that a comprehensive vision of ageing requires the exploration of potential transgenerational manifestation of senescence. In our study organism, the slight but detectable decline of egg size in some condition could well have such transgenerational consequences, especially given that we have shown that the smaller juveniles that hatch from smaller eggs survive less well in hash environmental conditions (Tully and Ferrière, 2008). It would be interesting to investigate the extent to which age-related maternal effects shape the offspring survival and actuarial senescence ("Lansing effect"), but also their long-term individual reproductive trajectories (reproductive senescence). Such "intergenerational transfer of ageing" could explain part of the non-genetic and non-environmentally induced phenotypic variability that we have observed between individuals of the same clone in the same environment (Monaghan et al., 2020).

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://zenodo.org/doi/10.5281/zenodo.10011211.

## Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

## Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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## Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1112045/full#supplementary-material

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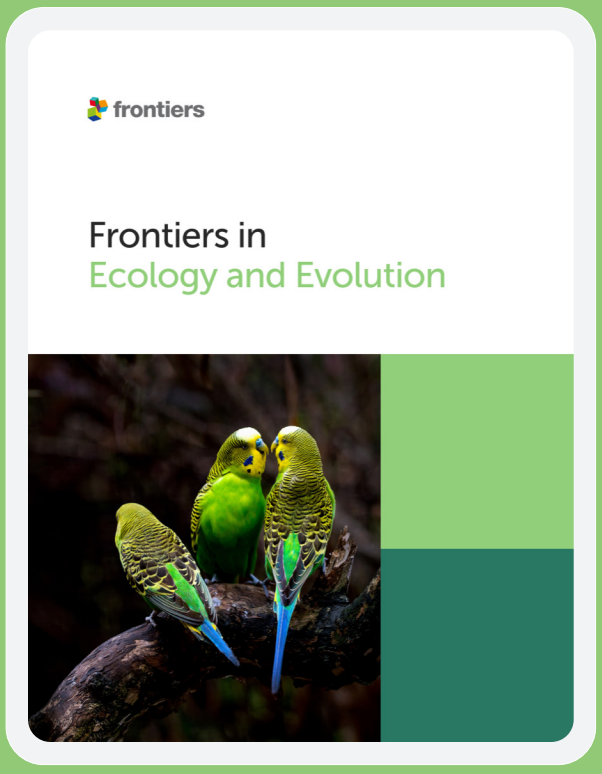


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