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New challenges and perspectives in conservation breeding programs

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New challenges and perspectives in conservation breeding programs

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Table of contents

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Editorial: New challenges and perspectives in conservation breeding programs

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Editorial on the Research Topic
New challenges and perspectives in conservation breeding programs

Faced with unprecedented extinction rates and escalating human impact on our planet, Conservation Breeding Programs (CBPs) remain the utmost tools to preserve animal biodiversity. This Research Topic aimed to bring together studies that deal with CBPs from different perspectives. Nine manuscripts have been published in this Research Topic encompassing different approaches (both *ex-situ* and *in-situ*) and animal species—primarily mammals, birds, and amphibians—in a variety of ecosystems spanning the Northern and Southern Hemispheres.

For an alarming number of animal species, CBPs represent the last line of defense against extinction. The ʻAlalā (*Corvus hawaiiensis*), the last endemic Hawaiian corvid, exemplifies this, surviving solely through captive breeding. Given its monogamous nature, mate selection and pair duration are pivotal for reproductive success in captivity. Using data recorded during four breeding seasons, Barrett et al. revealed that age, rather than pair duration, strongly influences reproductive outcomes, providing crucial insights for enhancing ʻAlalā CBPs.

A major factor that undermines CBPs' success is the limited understanding of animal reproductive biology and behavior, which remains well-documented for only a fraction of species, predominantly mammals (Wildt et al., 2010). In this Research Topic, Van Sluys et al. present the first ethogram of the critically endangered Plains-wanderer (*Pedionomus torquatus*), a ground dwelling bird endemic to Australia, paving the way for improved conservation management and husbandry of this species. Understanding the behavior of endangered species in their natural habitat is crucial for developing effective conservation strategies, a principle illustrated by the successful management of the giant panda (*Ailuropoda melanoleuca*). This iconic species serves as an example of a holistic approach in which husbandry practices align with animal behavior and physiology, which notably improve captive reproductive success from mating till cub rearing (Martin-Wintle et al., 2019; Ming-yue et al., 2021). These efforts contributed to the giant panda's downlisting from "Endangered" to "Vulnerable" on the International Union for

Conservation of Nature Red List. Collectively, these studies underscore the importance of animal behavior as a foundation for effective captive management.

When faced with small populations and low reproductive success in CBPs, assisted reproductive technologies (ARTs) associated with biobanking represent valuable tools to overcome fertility issues (Holt and Comizzoli, 2022). This is particularly relevant for amphibians, which are experiencing severe declines due to habitat degradation, population fragmentation, and diseases like chytrid fungus. In this interesting mini review, Silla and Byrne evaluate how ARTs may affect individual traits throughout amphibian life-stages, providing a "best-practice" framework for practitioners to assess the impact of protocol refinement on individual and population fitness. Additionally, targeted genetic intervention, such as promoting chytridiomycosis resistance, offers a novel conservation strategy for amphibian CBPs (Kosch et al., 2022).

Establishing ARTs requires optimized gamete collection protocols and anesthetic procedures that maximize gamete quality and quantity, while minimizing animal stress. Baquerizo et al. demonstrated that tiletamine hydrochloride plus zolazepam (Telazol®) yielded superior ejaculates in cheetahs (*Acinonyx jubatus*) undergoing electroejaculation compared to medetomidine, butorphanol, and midazolam. Similarly, in the Baw Baw frog (*Philoria frosti*), Gibert et al. found that gonadotrophin releasing hormone analogue (GnRHa) resulted in a higher proportion of offspring that reaches metamorphosis compared to GnRHa combined with metoclopramide. With robust physiological knowledge, ARTs like ovum-pick up, *in vitro* fertilization, and embryo transfer can be effectively implemented, sometimes leveraging protocols from domestic animals. However, in very small and infertile populations, ARTs might not be sufficient alone to support a viable and self-sustaining population. Under these circumstances, advanced cellular biotechnologies like somatic cell nuclear transfer, *in vitro* gametogenesis or gene editing might be necessary, raising significant ethical and welfare considerations (Korody and Hildebrandt, 2025). Interestingly, beyond species conservation, wildlife reproductive sciences can also contribute to human reproductive medicine, given shared reproductive features and challenges (Comizzoli et al., 2018). Wildlife reproductive success is affected by threats like those impacting human and livestock fertility, such as oxidative stress (Pintus and Ros-Santaella, 2021) and antimicrobial resistance (Doyle et al., 2025). Furthermore, reproductive sciences play a role in managing overabundant or invasive species, which pose a significant threat to biodiversity. Hormone- and immune-contraception are currently the least invasive and most ethically acceptable methods for wildlife and zoo population management (Asa and Moresco, 2019).

The burgeoning field of microbiota research is now providing new vital insights into wildlife health and welfare, knowledge that is essential for optimizing captive management practices and maximizing the health and survival of animals after reintroduction into the wild (Dallas and Warne, 2023). Maly et al. revealed significant differences in fecal microbiota between cheetahs from Namibia and the USA, with Namibian samples exhibiting greater bacterial diversity. The variation in microbial diversity between populations may help to understand the incidence of gastrointestinal and other diseases in captive cheetahs and to increase their survival and breeding success.

The goal of CBPs is to establish genetically diverse and demographically stable populations in their natural habitats, often through combined *ex-situ* and *in-situ* approaches. However, release outcomes are often jeopardized by high mortality, even when captive breeding is successful. To mitigate this, Nelson et al. developed a multi-step approach for the greater sage-grouse (*Centrocercus urophasianus*), which assesses landscape risk factors prior to translocation to maximize survival. As a promising alternative to traditional reintroduction methods, Galindo et al. illustrate the potential of embryo transfer as a valuable strategy to increase genetic diversity and minimize disease spread in translocated marsh deer (*Blastocerus dichotomus*). For more integrated conservation strategies, Staerk et al. developed a comprehensive and flexible decision-tree framework, balancing investment in habitat protection and captive breeding. To evaluate framework's effectiveness, the authors analyzed species composition and population size of 847 terrestrial vertebrates housed in European Union zoos, revealing that a significant proportion require further investment in captive breeding.

In conclusion, CBPs have been essential for safeguarding several mammals, birds, and amphibians. However, there is an urgent need to preserve other vertebrates like reptiles or fish, but also invertebrates like insects or mollusks, which account for the greatest proportion of animal species on our planet and in which biodiversity loss is equally dramatic (Cowie et al., 2022; Cox et al., 2022). Future efforts should prioritize establishing CBPs for these underrepresented taxonomic groups. A multidisciplinary approach, integrating wildlife biology with conventional and cutting-edge technologies, is crucial for successful CBPs. Importantly, habitat restoration and protection remain as fundamental as CBPs for the effectiveness of any conservation intervention.

Author contributions

EP: Writing – original draft. WH: Writing – review & editing. PC: Writing – review & editing. JR-S: Writing – review & editing.

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A retrospective analysis investigating the effects of Telazol® and medetomidine on ejaculate characteristics in cheetahs (*Acinonyx jubatus*)

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Zoo managed cheetahs provide an insurance population for wild cheetahs that are under threat of extinction from habitat loss, lack of prey, competition, pet trade and poaching for skin and bones. Assisted reproductive techniques including artificial insemination, *in vitro* fertilization, and embryo transfer augment natural breeding programs but rely on good quality semen for best results. It is understood that anesthesia can affect semen characteristics such as ejaculate volume, total sperm count, sperm motility, and incidence of urine contamination. Thus, the aim of this study was to conduct a retrospective analysis of 23 years of data to investigate sperm parameters of semen collected under anesthesia using medetomidine in combination with butorphanol and midazolam or Telazol® alone. Electroejaculation records (Medetomidine, Butorphanol, and Midazolam anesthetized n = 59 ejaculates, from 30 cheetahs, Telazol® anesthetized, n= 169 ejaculates, from 72 cheetahs) were evaluated for incidence of urine contamination. Electroejaculation records (Medetomidine, Butorphanol, and Midazolam anesthetized n = 21 ejaculates, from 17 cheetahs, Telazol® anesthetized, n = 143 ejaculates, from 63 cheetahs) were evaluated for total sperm count, total motility, ejaculate volume, and testicle size. Telazol® treated cheetahs had a numerically higher total sperm count (Median ± SD: 42.58 ± 77.8 × 10^6 spermatozoa) compared to those treated with medetomidine (Median ± SD: 31.2 ±44.58 × 10^6 spermatozoa), and a significantly ($p < 0.05$) higher sperm motility (Median ± SD: 70.0 ± 9.71%) compared to medetomidine (Median ± SD: 53.0 ± 16.41%) treated cheetahs. The findings of this study indicate that medetomidine anesthesia results in significantly lower sperm motility and Telazol® anesthesia results in a higher total sperm count and motility, thus resulting in higher quality ejaculate. This information can aid in the veterinary management of the species when involved in genome resource banking and assisted reproductive technologies.

KEYWORDS

semen collection, alpha-2 agonists, electroejaculation, genome resource banking, cheetah (Acinonyx jubatus)

1 Introduction

The International Union for Conservation of Nature's Red List classifies cheetahs (*Acinonyx jubatus*) as a "vulnerable" species due to a declining population size resulting from habitat loss or fragmentation, poaching and/or lack of available prey (IUCN/SSC, 2007). Additionally, cheetahs have experienced significant population bottlenecks with subsequent inbreeding which has resulted in a lack of genetic diversity (O'Brien et al., 1983; O'Brien et al., 1985; O'Brien et al., 1986; O'Brien et al., 1987; Menotti-Raymond and O'Brien, 1993). To mitigate further loss of genetic diversity, cheetahs are housed under managed care to act as insurance policies for the declining wild populations. Institutions of the Association of Zoos & Aquariums carefully manage breeding as part of the Species Survival Plan® to maximize genetic diversity.

Assisted reproductive techniques (ART) such as artificial insemination (AI) (Donoghue et al., 1992), *in vitro* fertilization and embryo transfer (Crosier et al., 2020) are used to augment breeding programs and aim to ensure that genetically underrepresented individuals reproduce and contribute offspring to the managed population. The cryopreservation and storage of spermatozoa in genome resource banks have provided a repository of genetic material that can be used to genetically manage future cheetah populations (Wildt, 1997). However, cheetah semen has been characterized as having low numbers of morphologically normal spermatozoa and low sperm concentrations (Wildt et al., 1983; Crosier et al., 2007; Terrell et al., 2016). The high incidence of abnormally structured cells makes cheetah spermatozoa highly susceptible to cryo-induced damage (Crosier et al., 2006). As a result, the ongoing systematic collection and cryopreservation of spermatozoa is required to support the genetic management of cheetahs through ART.

Semen is collected from cheetahs by a technique known as electroejaculation (EEJ) which requires a surgical plane of anesthesia (Howard, 1993). It has been well documented that analgesics can alter sperm function in a range of species (Agirregoitia et al., 2006; Carrillo et al., 2016; Kottwitz et al., 2017) including the domestic cat (Zambelli et al., 2007) and have anecdotally been reported to influence the success of the semen collection. In zoos, cheetahs were historically anesthetized with a protocol involving drugs such as Telazol®, ketamine, or xylazine for health examinations (Penfold, personal communication). In recent years, this protocol has changed and medetomidine is the more commonly used anesthetic due to better desired physiological effects such as stable heart rate, respiratory rates, and quick recovery (Miller et al., 2003; Williamson et al., 2018). Therefore, the aim of the present study was to retrospectively investigate the effects of anesthesia protocols using Telazol® and medetomidine on semen characteristics.

2 Materials and methods

2.1 Cheetahs and criteria for data selection

Cheetahs were housed at White Oak Conservation (WOC) in Yulee, Florida from 1998-2021 or at the Cheetah Conservation Fund (CCF) in Namibia from 2002-2005. Captive cheetahs at WOC were fed a diet of commercially available beef, horse, venison, and/or pork meat products (Milliken Meat Products Ltd, or Carnivore Diet 10; Natural Balance Pet Foods Inc., Pacoima, CA) seven days a week, bones one day a week, and supplemented with whole rabbits when available. Wild cheetahs at CCF were fed combination of donkey, horse and game species with mineral supplementation as described previously (Crosier et al., 2006; Crosier et al., 2007). Animals had access to *ad libitum* water. Cheetahs were housed individually or as coalition groups and ranged from one to fifteen years in age. Medical records, stored in Species 360 Zoological Information Management System (ZIMS and reproductive summary reports generated by the South-East Zoo Alliance for Reproduction & Conservation and CCF, were reviewed (228 procedures between the years of 1998 and 2021). Incidence of urine contamination was recorded and compared between the two anesthesia protocols (MBMZ anesthetized n = 59 ejaculates, from 30 cheetahs, Telazol® anesthetized, n= 169 ejaculates, from 72 cheetahs). The effect of age on total sperm count was investigated in each treatment group (MBMZ anesthetized n = 29 ejaculates, from 22 cheetahs, Telazol® anesthetized, n= 162 ejaculates, from 71 cheetahs). To investigate the effect of anesthesia protocols on semen characteristics, ejaculates collected from healthy, sexually mature (≥2 years of age) (Maly et al., 2018) males, that were not contaminated with urine, were included in the data analysis (MBMZ anesthetized n = 21 ejaculates, from 17 cheetahs, Telazol® anesthetized, n= 143 ejaculates, from 63 cheetahs).

2.2 Anesthesia

Males were anesthetized using one of two protocols. Protocol 1 utilized a range of 0.030-0.035 mg/kg of Medetomidine, 0.18-0.22 mg/kg of Butorphanol, and 0.10-0.14 mg/kg of Midazolam (MBMZ) in captive WOC cheetahs. Protocol 2 utilized a range of 4 mg/kg to 6 mg/kg of tiletamine hydrochloride plus zolazepam (Telazol®) (Crosier et al., 2007) in wild CCF cheetahs and two captive WOC cheetahs. While isoflurane gas was used during some cheetah immobilizations, it was not applied until after the EEJ procedure ended.

2.3 Semen collection and characterization

Prior to semen collection, testes were measured with calipers and the total testes volume was calculated ($V = L \times W^2 \times 0.524$; where V= total volume, L = total length and W = width)[14]. Investigators conducting semen collections (L.M.P. and A.E.C.) were trained by the same researcher (Dr. Jo-Gayle Howard) and followed the same protocol for semen collection (Howard, 1993). In brief, feces were manually removed from the rectum and the prepuce was rinsed with sterile phosphate-buffered saline. The penis was extended from the prepuce and placed in a sterile collection cup (BD Falcon, Franklin Lakes, NJ, USA). To collect the semen, a Teflon rectal probe (1.6 cm in diameter with three longitudinal electrodes) and electro-stimulator were used to administer 10 stimulations at 2, 3 and 4 volts (Series I), 3, 4, 5

volts (Series II), and 5 and 6 volts (Series III) (Crosier et al., 2006). Semen was characterized as previously described (Crosier et al., 2006; Johnson et al., 2010). Data sheets were reviewed for testicular volume, semen volume, total sperm count, total motility, and presence of urine contamination. In brief, volume was measured using adjustable micropipettes (Pipetman Classic, Gilson Incorporated, Middleton, WI, USA). Sperm concentration was calculated by diluting semen 1:400 with water and counting the number of spermatozoa in a 10 µl volume using a hemocytometer (Daigger Scientific, Inc., Hills, IL, USA). Total sperm count was calculated by multiplying semen volume by the sperm concentration. Sperm motility (%) was subjectively assessed using phase-contrast microscopy (40 ×, Olympus B-Max Microscope, Olumpus Optical Co. Ltd) examining 5 µl aliquot of raw ejaculate at 37°C with a minimum of three separate fields of view examined. The pH was measured using pH indicator strips (EM Science, Gibbstown, NJ, USA). A sample was considered to be contaminated with urine if the pH was <7.5 (Bertschinger et al., 2008).

2.4 Statistical analysis

Statistical analyses were performed using SigmaPlot (V15.0, Systat Software Inc. San Jose, CA, USA). Ejaculate characteristics between anesthesia protocols were analyzed by a one-way ANOVA and nonparametric Kruskal-Wallis Test. Spearman's correlation was used to evaluate the correlation between testicle size and total sperm count within anesthesia protocols. Incidence of urine contamination between anesthesia protocols was analyzed using a Pearson chi-squared test. Data are reported as the Median ± SD. Data was considered significant if $p < 0.05$.

3 Results

3.1 Age v. total sperm count

Analysis of n = 29 MBMZ treated ejaculates showed a decline in total sperm count with age (Figure 1A). Three males >10 years of age had a numerically lower total sperm count (mean ± SEM = 1.14 ± 1.21 × 10^6 spermatozoa) than sexually mature males ≤10 years of age (mean ± SEM = 44.38 ± 9.73 × 10^6 spermatozoa). Analysis of n = 162 Telazol® treated ejaculates also showed a decline in total sperm count with age (Figure 1B). Three males >10 years of age had a numerically lower total sperm count (mean ± SEM = 32.38 ± 9.69 × 10^6 spermatozoa) than sexually mature males ≤10 years of age (mean ± SEM = 67.99 ± 6.55 × 10^6 spermatozoa). These findings contributed to restricting sperm analysis data to sexually mature males ≤10 years of age.

3.2 Effects of anesthesia on semen collection

The incidence of urine contamination was greater ($p < 0.001$) for cheetahs anesthetized using MBMZ (50.85%) than Telazol® (4.14%). Although treatment groups were not statistically different, a numerically higher total sperm count was observed in cheetahs anesthetized with Telazol® (Median ± SD: 42.58 ± 77.8 × 10^6 spermatozoa) than MBMZ (Median ± SD: 31.2 ± 44.58 × 10^6 spermatozoa) (Figure 2). Total motility was significantly ($p < 0.05$) greater in ejaculates collected using Telazol® (Median ± SD: 70.0 ± 9.71%) than MBMZ (Median ± SD: 53.0 ± 16.41%) (Figure 3). There was a significant difference ($p < 0.05$) in testes size between both treatment groups (Median ± SD MBMZ: 11.53 ± 5.81 cm^3, Median ± SD Telazol®: 9.16 ± 2.9 cm^3) (Table 1). Testes size and total sperm count were not correlated in the MBMZ treatment group ($p > 0.05$; R = 0.086) but were weakly correlated in the Telazol® treatment group ($p < 0.05$; R = 2.1).

4 Discussion

This is the first study to examine the effect of anesthetic drugs on cheetah spermatozoa. The most compelling impact of anesthesia was on sperm motility, with significantly lower motile sperm found in the MBMZ protocol. Although the testes size was larger in cheetahs anesthetized with MBMZ versus Telazol®, and therefore might have been expected to produce more sperm (Olar et al.,

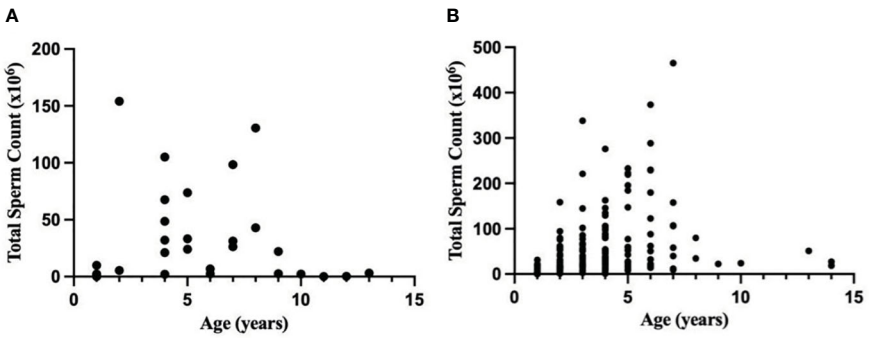

FIGURE 1
Relationship between age (years) and total sperm count (x10^6) in a normal ejaculate for healthy cheetahs (*Acinonyx jubatus*) anesthetized with **(A)** MBMZ (n = 29 ejaculates, 1-13 years old, three males > 10 years old included) or **(B)** Telazol® (n = 162 ejaculates, 1-14 years old, three males > 10 years old included).

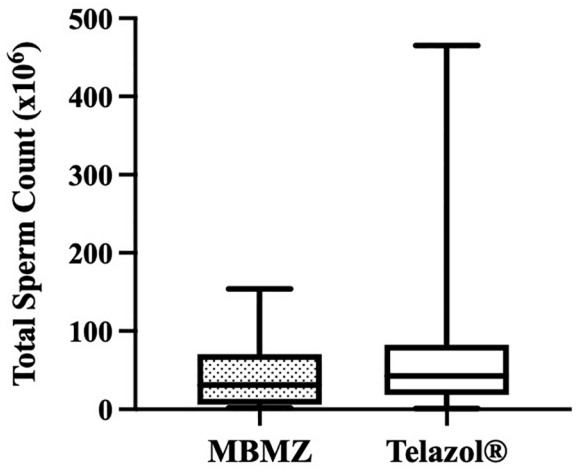

FIGURE 2
Box and Whiskers plot showing variation of total sperm count in cheetah (*Acinonyx jubatus)* ejaculates collected by EEJ using MBMZ or Telazol® anesthesia protocols (MBMZ anesthetized n = 21 ejaculates, from 17 cheetahs, Telazol® anesthetized n = 143 ejaculates, from 63 cheetahs). Boxes enclose the 25th and 75th percentiles, the horizontal bars within the boxes are the median values, and the whiskers extend to the minimum and maximum values observed.

1983), the Telazol® treated cheetahs resulted in a greater total sperm count and significantly greater motility. Correlation coefficients reveal that in the MBMZ protocol, testes size was not correlated with total sperm count ($p > 0.05$; $R= 0.086$), however, in the Telazol® protocol, testes size and total sperm count were weakly correlated ($p < 0.05$; $R = 2.1$). Total sperm count and motility are vital characteristics in determining the quality of sperm samples to be cryopreserved for use in artificial breeding. The incidence of urine contamination was characterized during semen collection and was more commonly observed in the MBMZ protocol.

Medetomidine is an alpha-2-adrenergic agonist commonly used in non-domestic felid anesthesia (Brown et al., 1991). Alpha-2 receptors are found along the male reproductive tract and are required for successful emission and ejaculation (McDonnell, 1992). Studies investigating the effects of medetomidine, dexmedetomidine, or xylazine on the hypothalamic-pituitary axis demonstrated these drugs' ability to suppress anti-diuretic hormone secretion. Physiologically this limits water re-uptake by the kidneys, leading to increased volume for urination, seen most pronounced with xylazine (Rouch and Kudo, 1996; Cabral et al., 1998; Nuñez et al., 2004; Villela et al., 2005; Murahata and Hikasa, 2012; Uddin et al., 2021). Additionally, medetomidine targets alpha-2 receptors of smooth muscle in the bladder and urethra, possibly leading to conflicting contractions and urine contamination during semen

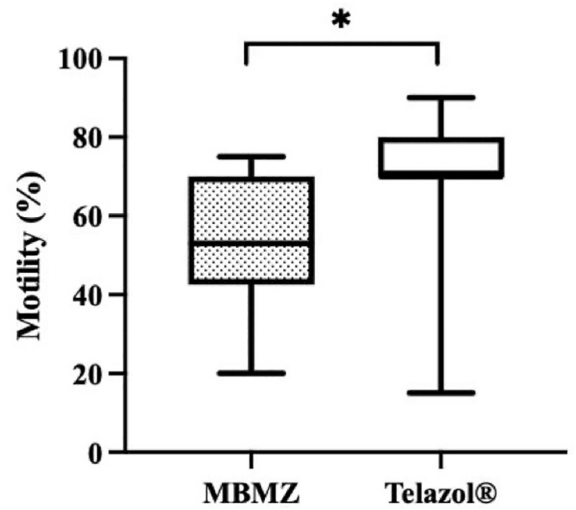

FIGURE 3
Box and Whiskers plot showing variation of motility in cheetah (*Acinonyx jubatus)* ejaculates collected by EEJ using MBMZ or Telazo® anesthesia protocols (MBMZ anesthetized n = 21 ejaculates, from 17 cheetahs, Telazol® anesthetized n = 143 ejaculates, from 63 cheetahs). Boxes enclose the 25th and 75th percentiles, the horizontal bars within the boxes are the median values, and the whiskers extend to the minimum and maximum values observed. The asterisk indicates a significant difference in motility ($p < 0.05$).

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TABLE 1 Ejaculation characteristics of cheetahs anesthetized with MBMZ and Telazol®.

Drug	MBMZ n = 21		Telazol® n = 143	
	Median ± SD	Mean ± SEM	Median ± SD	Mean ± SEM
Testes Volume (cm^3)	11.53 ± 5.81[a]	14.21 ± 1.27	9.16 ± 2.9[b]	9.62 ± 0.25
Semen Volume (ml)	1.08 ± 0.93[a]	1.21 ± 0.21	2.71 ± 1.24[b]	2.69 ± 0.1
Total Sperm Count ($\times 10^6$ sperm)	31.2 ± 44.58[b]	44.38 ± 9.73	42.58 ± 77.8[b]	67.99 ± 6.51
Motility (%)	53.0 ± 16.41[a]	53.35 ± 3.58	70.0 ± 9.71[b]	72.17 ± 0.81

Superscripts ([a,b]) indicate a significant difference ($p < 0.05$). Superscripts ([b,b]) indicate no significant difference.

collection (Michel and Vrydag, 2006). Smooth muscle contractions along the male reproductive tract are required to mediate ejaculation, but the reproductive physiology remains complex (McDonnell, 1992). Past studies in domestic species have demonstrated that alpha-2 agonists at higher doses result in increased sperm concentration and motility (Da Silva et al., 2021), but do not stimulate the accessory glands adequately, resulting in a lower volume of semen (McCue, 2021). Incidence of retrograde flow of semen involving alpha-2's has also been observed (Dooley et al., 1990; Zambelli et al., 2007) though positive effects on sperm concentration were noted with alpha-2's administered at higher doses. Urine contamination, retrograde flow, and low sperm volume can result in poor sperm quality which is problematic for assisted reproductive techniques and genome resource banking.

The other two components of Protocol 1 were butorphanol (an opioid) and midazolam (a benzodiazepine). When used alone for sedation, butorphanol has been associated with high sperm concentration at collection, yet when combined with an alpha-2 agonist and NMDA antagonist, it has shown a decrease in sperm concentration (Ungerfeld et al., 2022). Midazolam is not commonly used as the sole choice for sedation. In a laboratory setting where rats were administered midazolam twice daily for a week, direct epididymal collections resulted in inferior sperm motility compared to collections from rats administered propofol (Celaleddin and Volkan, 2020). A protocol limiting the use of alpha-2 agonists at low doses might be better for semen collection and genome resource banking and remains a future area of research; additional information on the roles opioids and benzodiazepines play in semen collection is also warranted.

In contrast to medetomidine, Telazol® antagonizes N-methyl-D-aspartate (NMDA) receptors to produce anesthetic effects (Lester, 2012). Multiple studies of domestic and wild felids anecdotally support the notion that sperm motility has a high mean value when Telazol® was used (Brown et al., 1991; Howard et al., 1992; Long et al., 1996). In one study involving six domestic cats, researchers collected semen via EEJ methods using Telazol® anesthesia resulting in an average sperm motility of 82.1% (Long et al., 1996). Similarly, semen also was acquired from six male cheetahs using EEJ methods and Telazol®, with an average motility of 75% (Howard et al., 1992). Lastly, in six adult and four young adult Serengeti-Plains lions under the same conditions (using EEJ and Telazol®), the average motility was 89% and 72%, respectively (Brown et al., 1991). In the present study, spermatozoa were 70.0 ± 9.71% motile in ejaculates from cheetahs treated with Telazol®.

A numerical decline in sperm concentration with age was observed. Males over 10 years old produced a lower total sperm count. These findings are in agreement with other studies where a decrease in litter numbers for males greater than or equal to 13 years of age (Bertschinger et al., 2008) was seen suggesting lower total sperm count as a possible explanation for a decline in sperm quality.

Qualification of cheetahs and population sites were limitations to this study. As previously stated, the original 228 semen samples did not all qualify for final statistical analysis based on male prepubescence, senescence (Durrant et al., 2001; Maly et al., 2018), urine contamination, or disease. Additionally, data from some cheetahs were not considered due to administration of additional drugs or isoflurane gas during semen collection. Some medical records lacked enough information to include as well. Finally, there was a limitation of unbalanced design in treatment groups between populations, as two captive cheetahs from White Oak Conservation were treated with Telazol® and grouped accordingly. In an attempt to present the most vital information, sample sizes were limited. Moving forward, detailed medical records are essential to retrospective analyses that inform reproductive physiologists and veterinarians on protocols conducive to adequate ejaculate quality. Additionally, the two cheetah populations are functionally different. Though unavoidable in retrospect, differences include rangeland (wild vs. captive), diet (carcass vs. frozen and prepared), and management. A difference in testes size was appreciated statistically between the two populations in this study attributed to these differences. In an earlier study, a difference in semen quality was not observed in captive versus wild populations of cheetahs (Wildt et al., 1987); a difference cannot be determined as our design is unbalanced (animals treated with Telazol® were both captive and wild). To confirm a difference, controlled studies using MBMZ in wild and captive populations are necessary to compare.

The present study emphasizes that anesthetic protocol should be considered when semen collection of genome resource banking is the primary reason for sedation of endangered felid species. The results inform veterinarians and reproductive physiologists of the effects alpha-2 agonists have on semen collection procedures in cheetahs. With the availability of reversible medications paired with alpha-2 agonists and smoother recoveries, it is unlikely that Telazol® will be used in the future, but manipulating dosages for medetomidine could

be considered. The retrospective analysis of collections in cheetahs adds to the unique history zoological institutions share in managed breeding and warrants continued research to better existing protocols.

Data availability statement

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

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because this is a retrospective study on cheetahs.

Author contributions

CB: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. LP: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing. JG: Data curation, Formal Analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing. SC: Investigation, Methodology, Resources, Writing – review & editing. LM: Data curation, Investigation, Methodology, Resources, Writing – review & editing. AC: Data curation, Investigation, Methodology, Resources, 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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A decision framework to integrate *in-situ* and *ex-situ* management for species in the European Union

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Zoos and aquaria in the European Union (EU) can play a crucial role in the conservation of EU species, as they currently hold nearly half (49%) of EU terrestrial vertebrates. In this study, we analyzed the species composition and population sizes of EU zoos and developed a framework to prioritize recommendations for additional *ex-situ* and *in-situ* interventions for 277 at-risk EU species. Our results showed that EU zoos currently hold 39% of threatened EU species, 27% of EU endemic species, 62% of EU species vulnerable to climate change, 20% of EU species listed by the Alliance for Zero Extinction (AZE), 25% of Evolutionary Distinct and Globally Endangered (EDGE) EU species, while only 5% are subject to *ex-situ* conservation. Using our framework, we found that additional captive breeding was recommended for 60-61%% of species while expanding protected areas was recommended for only 2–22%, as 217 out of 277 species already met habitat protection targets. Both interventions were recommended for up to 20% of species, while the remaining 18% required no interventions because captive populations and habitat protection fully met targets. Our flexible framework can support more effective integrated conservation planning decisions for EU species and help identify target species for further in-depth assessment by the IUCN *Ex-situ* guidelines.

KEYWORDS

captive breeding, conservation planning, cost-effectiveness, zoos, prioritization, Natura2000, Species360, EU zoos directive

1 Introduction

Despite concerted efforts to conserve biodiversity, 14% of mammals, 18% of birds, 21% of reptiles, and 22% of amphibians in the European Union (EU) are at risk of extinction (Temple and Terry, 2007; Cox and Temple, 2009; Temple and Cox, 2009; BirdLife International, 2021) due to habitat loss and fragmentation, pollution, over-exploitation, invasive species, and climate change (European Commission, 2020). Although initiatives like the EU Biodiversity Strategy 2030 (European Commission, 2020) aim to halt habitat loss and species decline by expanding and restoring the EU network of protected areas through Natura 2000 sites (Council Directive 92/43/EEC, 1992; Directive 2009/147/EC, 2009), it is apparent that much work remains to be done. While the legal protection of habitats has shown promise in increasing population trends for some bird and mammal species (Littlewood et al., 2020; Williams et al., 2020), many habitats are still insufficiently protected (Maiorano et al., 2015), and some are continuing to deteriorate (European Commission, 2015).

To ensure the long-term survival of terrestrial vertebrates in the EU (EU species, hereafter), habitat protection alone may not be sufficient for some species. An integrated conservation approach that includes *ex-situ* programs is required to supplement habitat protection efforts. *Ex-situ* conservation measures can be crucial for ensuring species survival until threats are mitigated in the wild (IUCN/SSC, 2014). According to Bolam et al. (2021) *ex-situ* conservation has contributed to preventing the extinction of at least 20 out of 32 bird species and 9 out of 16 mammal species that might otherwise have faced extinction between 1993-2020. Therefore, it is essential to prioritize species that require *ex-situ* conservation and assess the potential role of zoos and aquariums in supporting these programs. Conservation organizations, such as the International Union for Conservation of Nature (IUCN), include *ex-situ* action recommendations in their assessments for the Red List of Threatened Species. The IUCN Conservation Planning Specialist Group proposes the integration of all populations of a species, including *ex-situ* animals, into a single management plan, as outlined in their One Plan Approach (Byers et al., 2013). This approach involves protecting populations in natural habitats and implementing *ex-situ* measures such as captive breeding, assisted colonization, and genetic rescue programs.

European zoos and aquariums are invaluable resources in the field of conservation, providing a wealth of expertise in captive breeding, animal husbandry, veterinary care, and reintroduction programs. The EU Zoos Directive recognizes the critical role that these institutions play in conservation efforts and encourages alignment with existing policies, such as the EU Birds and Habitats Directives (Sikkema et al., 2015). The European Association of Zoos and Aquaria (EAZA) supports the integration of multiple directives to promote conservation efforts (EAZA, 2017). To support the strategic assessment of species in need of *ex-situ* conservation, the IUCN Species Survival Commission developed *Guidelines on the Use of Ex situ Management for Species Conservation* (IUCN *Ex-situ* guidelines, hereafter; IUCN/SSC, 2014). These guidelines provide a framework for informed decision-making for *ex-situ* management based on five key steps: 1) review of species status and threats, 2) evaluation of the role of *ex-situ* interventions for species conservation, 3) assessment of biological factors and practical considerations, 4) appraisal of feasibility, risks and resources, and 5) transparent decision-making. Although the *Ex-situ* guidelines are applied to regional or global collection planning in CPSG's Integrated Collection Assessment and Planning (ICAP) workshops, assessing all 847 terrestrial vertebrates in the EU would require significant resources. Therefore, the development of quantitative tools to assist conservation planners in moving more efficiently from assessment to planning would greatly benefit *ex-situ* conservation efforts. Efficient conservation planning relies on prioritization approaches that explicitly consider probabilities of success, risks, and costs involved. Numerous studies have demonstrated the effectiveness of these approaches, for example, in the context of conserving Australian biodiversity (Evans et al., 2015) and managing threatened species in New Zealand (Joseph et al., 2009). In addition, such approaches offer a valuable mathematical framework that could support experts in prioritizing species for the assessment against the IUCN *Ex-situ* guidelines.

This study has two primary objectives. Firstly, it aims to provide a baseline assessment of the current and potential contribution of EU zoos toward *ex-situ* management and species conservation at large. Secondly, it aims to develop a comprehensive framework to prioritize EU threatened species for the in-depth implementation of IUCN *Ex-situ* guidelines. To achieve this, we propose a robust multiple objective prioritization framework that incorporates various factors into a decision tree. These include the species' extinction risk probabilities *in-situ* and *ex-situ*, evolutionary distinctiveness, and estimated costs of captive management and habitat protection. This framework allows deriving decisions with and without budget constraints, incorporating estimated costs. We conducted extensive sensitivity analyses to evaluate the impacts of model assumptions and decision-makers' value judgments on the prioritization outcomes.

By adopting this proposed framework, our study aims to identify species for in-depth assessment that warrant further evaluation in accordance with the IUCN *ex-situ* guidelines. This framework will provide valuable support to EAZA Taxon Advisory Groups, Regional Collection Planners, EU policymakers, and conservation practitioners, facilitating the rigorous application of prioritization strategies for species conservation management.

2 Materials and methods

2.1 Datasets

To underpin our analyses, we collated several datasets related to EU species conservation both within zoos (*ex-situ*) and in the wild (*in-situ*). We initially considered 847 EU terrestrial vertebrates (i.e., mammals, birds, reptiles, and amphibians), defined as species regionally assessed within the EU based on the European IUCN Red List of Threatened Species, excluding those categorized as 'Not Evaluated', 'Extinct' or 'Possibly Extict' (Temple and Terry, 2007; Cox and Temple, 2009; Temple and Cox, 2009; BirdLife

International, 2021). We limited our analysis to terrestrial vertebrates due to the lack of data on habitat protection, vulnerability to climate change, and evolutionary distinctiveness for aquatic, invertebrate, and plant taxa.

To assess the contribution of zoos to EU species conservation, we quantified the population sizes of the 847 EU species housed in zoos and aquariums in the European Union using the Species360 Zoological Information Management System (ZIMS; Species360, 2021). ZIMS is the most comprehensive animal care real-time database in which >1,200 aquariums and zoos share standardized data on 22,000 species across 96 countries. Our analysis focused on data from 481 Species360 member institutions located within EU countries (EU zoos hereafter). These institutions, including all zoos and aquariums accredited by the EAZA, use ZIMS for their record-keeping purposes.

To compare population sizes between threatened and non-threatened species in EU zoos, we used a quasi-Poisson regression model that accounts for overdispersion, with log-link (Equation 1):

$$\mathrm{Log}\,(\mu_i) = \beta_0 + \beta_1 H_i + \beta_2 T_i \quad (1)$$

where $E(N_i) = \mu_i$ for all $I = 1, 2,\ldots, n$, where n is the total number of species. Here N_i represents the population size, calculated as the sum of all individuals across EU zoos, and H_i corresponds to the taxonomic class (i.e., mammals, birds, reptiles, or amphibians) of each species *i*. T_i serves as an indicator denoting whether species *i* is categorized as threatened according to the EU Red List, i.e. listed as Vulnerable (VU), Endangered (EN) or Critically Endangered (CR), or as non-threatened.

To determine the representation of EU species in *ex-situ* management programs we established the following criteria: i) inclusion in an EAZA *Ex-situ* Programme (EEP) or a European Studbooks (ESB) program; ii) presence in the amphibian *ex-situ* monitoring from the Amphibian Ark (Amphibian Ark, 2019); or iii) inclusion in other *ex-situ* programs documented on the IUCN Red List. Note that EEPs are the most intensively managed breeding programs, and ESBs are typically for species of lower conservation priority (EAZA, 2019).

We cross-referenced our list of EU species held in zoos with different prioritization assessments: i) the Alliance for Zero Extinction database, which identifies species listed as CR or EN and confined to a single remaining site (Alliance for Zero Extinction (AZE), 2010); ii) the Evolutionary Distinct and Globally Endangered list, which identifies species that are both phylogenetically unique and globally endangered based on the IUCN Red List (EDGE, Gumbs et al., 2018); iii) species endemism within Europe and the EU (Temple and Terry, 2007; Cox and Temple, 2009; Temple and Cox, 2009; IUCN, 2019; BirdLife International, 2021), and iv) species categorized as vulnerable under the IUCN Climate Change Vulnerability Assessment (Carr, 2011; Foden et al., 2013). The latter assessment utilizes trait-based analysis, considering species' exposure, sensitivity, and adaptability to climatic changes. It is available for all classes except mammals, and species are categorized with either 'High' or 'Low' vulnerability. For few species with missing data on evolutionary distinctiveness or climate change vulnerability, we used data of closely related species.

Next, we applied our prioritization framework (see section 2.2.) to a subset of 277 at-risk species out of the initial 847 EU species. We defined at-risk species as those considered threatened by the EU Red List (CR, EN, or VU) or identified as highly vulnerable to climate change, even if listed as Least Concern (LC) or Near Threatened (NT). We included climate change vulnerable species because the IUCN Red List criteria may not fully capture all future risks associated with climate change (Akçakaya et al., 2006 but see Keith et al., 2014). Moreover, *ex-situ* conservation interventions may be particularly valuable for species threatened by climate change (Shoo et al., 2013). Given the critical role of endemic species in conservation efforts, we further expanded our scope to include Near Threatened species endemic to Europe, provided that the species geographic range was at least partially within the EU. Our analyses were limited to species with available estimates of habitat availability and coverage by protected areas (Maiorano et al., 2015), which led to the exclusion of 33 species (excluded species are indicated in the Supplemental Material).

To integrate the datasets, we standardized species nomenclature according to the Global Biodiversity Information Facility (GBIF Secretariat, 2019) using the *taxize* package in R (Chamberlain and Szöcs, 2013). In a few cases where names could not be found (e.g., because species were only recently described), we used the Red List taxonomy. All calculations and analyses for this study were performed in R version 3.6.1 (R Core Team, 2017). The combined and standardized dataset is available in the Supplemental Material.

2.2 Prioritization framework

We developed a project prioritization framework based on Joseph et al. (2009) with multiple objectives: minimizing extinction risk, maximizing contributions to evolutionary diversity, and minimizing conservation management costs for the largest possible number of EU threatened species over a 100-year timeframe. First, we constructed a decision tree to determine, whether each species *i* should undergo conservation intervention *j* based on the net expected benefit (B_{ij}) associated with that intervention. We then calculated the cost-effectiveness of each intervention by calculating the ratio of potential benefits to costs (C_{ij}). To evaluate the relative importance of species conservation projects we incorporated benefits, costs, and species contributions to evolutionary distinctiveness into a ranking criterion (R_i) for each species. This ranking criterion is based on the Noah's Ark framework (Metrick and Weitzman, 1998) and given by the equation:

$$R_i = \max\left(\frac{B_{ij}}{C_{ij}}\right) D_i \quad (2)$$

where, for each species *i*, the maximum cost-effectiveness between the different management interventions *j* was weighted by the species evolutionary distinctiveness, D_i, obtained from Gumbs et al. (2018). To ensure comparability, we ranked each taxonomic class separately because not all data used to calculate R_i were available for each class. We further scaled the range of D_i to match that of the benefits (0–1) by dividing D_i values by the

maximum D_i within each taxonomic class. For two species with missing evolutionary distinctiveness data, we assigned the average D_i score of their respective genera.

Given potential variability in decision-makers' values, we explored four variations of the ranking criterion: (i) based on benefits only (R_{i1}), (ii) benefits weighted by evolutionary distinctiveness (R_{i2}), (iii) cost-effectiveness (R_{i3}), and (iv) weighted cost-effectiveness (R_{i4}, Equation 2). By including these options, we aimed to capture different perspectives and preferences in the decision-making process.

2.3 Benefits of conservation interventions

For each species, we considered two conservation interventions: invest in habitat protection ($W = 1$) or no investment ($W = 0$) and invest in captive breeding ($Z = 1$) or no investment ($Z = 0$). These interventions were considered as additional actions assuming that current levels of protection and zoo populations are maintained. Thus, the decision tree (Figure 1) presented four options represented by branches diverging from decision nodes (squares): (i) invest in habitat protection and captive breeding ($W = 1, Z = 1$), (ii) invest in habitat protection only ($W = 1, Z = 0$), (iii) invest in captive breeding only ($W = 0, Z = 1$) or (iv) do nothing ($W = 0, Z = 0$).

We defined habitat protection as protecting species habitat through the establishment of protected areas (IUCN categories I–VI) or Natura 2000 sites, according to the representation target set by Maiorano et al. (2015) for each species in the EU. These targets were based on a proportion of the species' range, considering whether the species had a narrow or wide distribution. We defined captive breeding as maintaining a captive insurance population of at least 100 individuals across EU zoos in ZIMS, assumed to sustain 90% of a population's genetic diversity for 100 years (Soulé et al., 1986; Lees and Wilcken, 2009).

The benefit (B_{ij}) of a conservation intervention is the difference in species' persistence probability with and without the intervention. To calculate the persistence probability under each intervention scenario, we developed probability models of species persistence and extinction in the wild or in captivity, depicted by the chance nodes (circles) in Figure 1. The species' persistence probability was determined by the function $P_{ik}(.)$, where index $k = 1,2$ referred to species persistence in the wild ($k = 1$) or in captivity ($k = 2$).

In the wild, we assumed that a species' persistence probability would vary with its IUCN Red List status and vulnerability to climate change. To calculate this, we transformed these originally categorical variables into probabilities. For the IUCN Red List categories, we adopted probability values based on the IUCN criterion E formulations with projected extinction probabilities to 100 years (LC = 0.0001, NT = 0.01, VU = 0.1, EN = 0.667 and CR = 0.999; Mooers et al., 2008). For species with high vulnerability to climate change, we assigned a fixed extinction probability of 0.3, except for mammals, where these data were unavailable. To explore

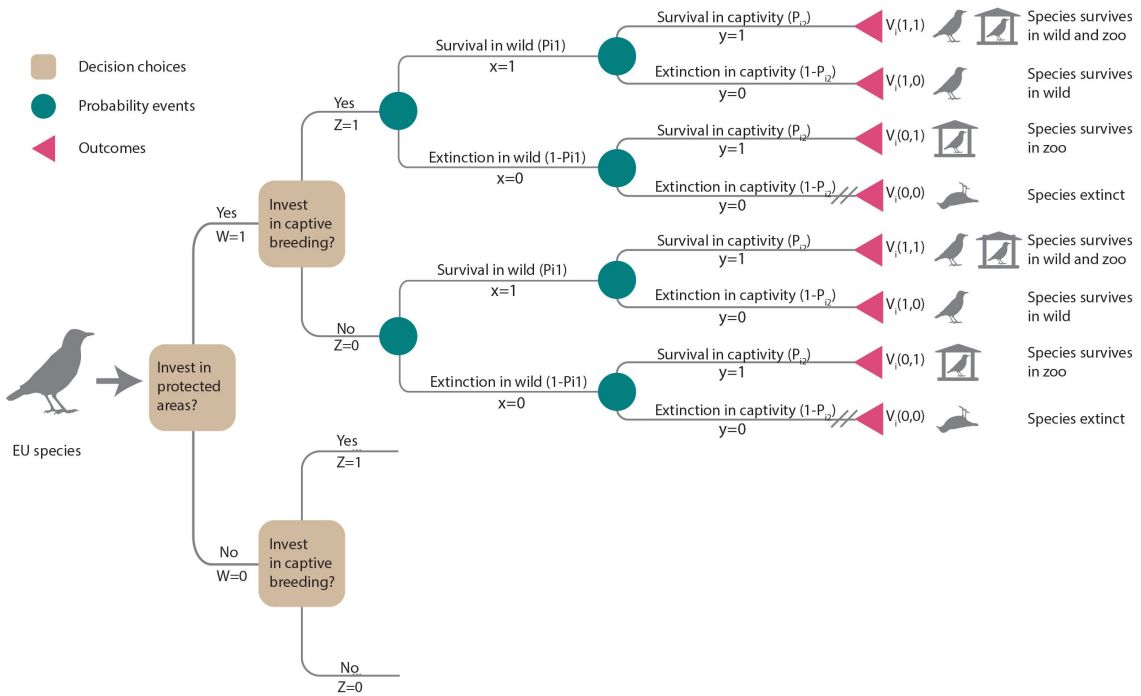

FIGURE 1
Decision tree illustrating the choices of additional investments in habitat protection (W = 1) or not (W = 0) and captive breeding (Z = 1) or not (Z = 0). Each decision choice (squares) leads to consecutive chance nodes (circles) describing uncertain events that may result in species persistence or extinction in the wild or in captivity, respectively. Outcomes (triangles) terminate each path. Cross-hatching (//) indicates the least preferred outcome (i.e., extinction). Outcomes are weighted by decision-makers' preferences for that outcome, $V_i(x,y)$. The expected value for each of the four options is the sum of its probabilities weighted by $V_i(x,y)$. Lower branches are the same as upper branches and were not illustrated.

10.3389/fcosc.2023.1298850

how climate change-related extinction probabilities influenced our results, we conducted a sensitivity analysis varying the range of values from 0 to 0.5.

In scenarios where no additional investments were made in habitat protection (W = 0), we calculated persistence probability in the wild for species i as in Equation 3:

$$P_{i1}(W, I_i, C_i) = (1 - I_i)(1 - K_i) \tag{3}$$

where I_i represents the extinction probability based on EU Red List categories and K_i represents the extinction probability based on climate change vulnerability, assuming these probabilities were independent.

On the other hand, if additional investments were made in habitat protection (W = 1), persistence probability was calculated as in Equation 4:

$$P_{i1}(W, I_i,\ K_i) = (1 - I_i A_i)(1 - K_i A_i) \tag{4}$$

where A_i is the proportion of a species' target area currently under protection (Maiorano et al., 2015). We assumed that the current persistence probability of the species would scale linearly with the proportion of the remaining unprotected target area that we choose to invest in and protect. The smaller the proportion of the target area already protected, the greater the potential increase in persistence probability when investing in habitat, assuming that habitat that is already protected will stay safeguarded over the next 100 years. If this proportion is 1 (i.e., the target is fully met), then the overall persistence probability is the same as if we decide to do nothing $P_{i1}(W, I_i, K_i)$. Under human care, we assumed that species persistence probability would be influenced by its breeding success (G_{il}), where index l = 1,2,3,4 refers to captive breeding expertise. Based on Conde et al. (2015), we assumed that if a species or congeneric species is present within zoos, management expertise would be available for that species. We categorized levels of management expertise as follows: G_{i1}: No expertise exists and neither species i nor a congeneric species are kept in a zoo, G_{i2}: transferrable expertise exists, a congeneric species is kept in a zoo, implying some level of knowledge transfer and shared management practices, G_{i3}: expertise exists but may be difficult to access because the species is kept in a zoo outside the EU, and G_{i4}: the species is kept in EU zoos, implying that management expertise is available.

Based on the level of management expertise, we calculated persistence probability in captivity for species i and management expertise level l as in Equation 5:

$$P_{i2}(Z, G_{il}) = \begin{cases} 0.05 & \text{for } Z = 1 \text{ and } G_{i1} \\ 0.1 & \text{for } Z = 1 \text{ and } G_{i2} \\ 0.2 & \text{for } Z = 1 \text{ and } G_{i3} \\ 0.2 + 0.8Q_i & \text{for } Z = 1 \text{ and } G_{i4} \\ Q_i & \text{for } Z = 0 \end{cases} \tag{5}$$

For case G_{i4} (expertise within EU zoos), we assumed that persistence probability should increase as a function of the ratio Q_i (i.e., the proportion of individuals available with respect to viable population size), given by Equation 6:

$$Q_i = \min\left(1,\ \frac{N0_i}{Nv_i}\right) \tag{6}$$

where $N0_i$ is the number of individuals in EU zoos and Nv_i is the number of individuals to ensure a viable population for each species i. The last option, Z = 0, refers to not investing in captive breeding. Here, we assumed that the persistence probability only depended on Q_i. We conducted a sensitivity analysis for the chosen values of $P_{i2}(Z,\ G_{il})$ (see section 2.5). Species extinction probability was given as $1 - P_{i2}(Z, G_{il})$.

Each management option considered had the potential to lead to four possible outcomes for a species: persistence in the wild and in captivity, persistence only in the wild, persistence only in captivity, or extinction within the EU (see outcomes in Figure 1). Decision-makers can assign weights to reflect the desirability or value of each outcome. We let x be the outcome of the species in the wild, where $x = 1$ if the species is extant and $x = 0$ if extinct, and y be the outcome of the species in captivity, where $y = 1$ if extant and $y = 0$ otherwise. $V_i(x,y)$ is the value of each possible outcome for species i (Figure 1). Our decision-making framework prioritized species persistence in the wild as the highest priority, followed by maintaining *ex-situ* insurance populations. On a scale ranging from 0 to 1, we assigned a value of 0 to the outcome of species extinction ($V_i(0,0) = 0$) and the highest value was given to a species persisting in the wild or persisting both in the wild and *ex-situ* ($V_i(1,0) = V_i(1,1) = 1$). We assumed the decision-maker would place a higher value on species persisting *ex-situ* compared to a species becoming extinct ($V_i(0,1) = 0.1$). To assess the impact of value judgments (V_i) on prioritization, we conducted a sensitivity analysis (see section 2.5).

We calculated the expected value E_{ij} of each conservation intervention j as the sum of probabilities weighted by the corresponding value judgments V_i. For example, the expected value of securing a species in the wild and in captivity ($W = 1$, $Z = 1$) can be derived from the decision tree (Figure 1) and is estimated as in Equation 7:

$$\begin{aligned} E_{ij}(W = 1,\ Z = 1) = {} & P_{i1}P_{i2}V_i(1,1) + \\ & P_{i1}(1 - P_{i2})V(1,0) + \\ & (1 - P_{i1})P_{i2}V(0,1) + \\ & (1 - P_{i1})(1 - P_{i2})V(0,0) \end{aligned} \tag{7}$$

Finally, the benefit of a conservation intervention is the difference between the expected value of the intervention and the expected value of no intervention ($B_{ij} = E_{ij} - E_{i0}$).

2.4 Costs of conservation interventions

We estimated the cost of conserving species i under interventions W and Z as $C_i(W,Z)$. We obtained the species gap area (i.e., the area of a species' range currently not under protection) based on a representation target for each species (Maiorano et al., 2015). The cost estimation included both one-off land purchase costs and average annual recurrent costs of

nature management per km^2 across 28 EU countries, based on Verburg et al. (2017). Land purchasing costs for species gap areas included the cost of converting agricultural land to natural vegetation, using average cost of agricultural land prices across 28 EU countries (see Table 3.8 in Verburg et al., 2017). These were one-off costs at the start of the 100-year planning period. Recurrent management costs included nitrogen and conservation management costs (i.e., conservation of specific vegetation) as an average of 58.56 €/ha/year (Verburg et al., 2017, p. 52) for all land uses adjusted to the 100-year period by multiplying with 100. To obtain costs of protecting each species gap area, we multiplied the species gap areas with one-off and recurring costs per km^2. Gap areas can overlap between species, so protecting one species' habitat can benefit and reduce costs for others. Therefore, we calculated the proportion of overlap of gap areas among all species within each taxonomic class (i.e., because our prioritization framework ranks among species within each class). We divided the total cost per species by the sum of the overlap proportions with other species sharing the same gap area. For example, if a species shared 50% of its gap area with one other species and 25% with another, we divided the costs by 1.75.

We estimated the cost of captive breeding for each species, considering a population size of 100 individuals, following Conde et al. (2015). For mammals and birds, we estimated costs from a regression analysis of body mass (Balmford et al., 1996) with data from Pacifici et al. (2013) for mammals and Dunning (2007) for birds. For reptiles and amphibians, we estimated costs based on the Amphibian Ark Amphibian Conservation Action Plan (Gascon et al., 2007; see also Supplementary Table 1). The costs associated with both interventions (habitat protection and captive breeding) were calculated separately and then summed, assuming independence between the two.

2.5 Sensitivity analyses

To evaluate robustness of the overall conservation recommendations, we assessed the sensitivity of our model to three factors: value judgments, $V_i(x,y)$, extinction probabilities for climate-vulnerable species (K_i), and persistence probabilities based on management expertise, $P_{i2}(Z=1,G_{ik})$. We calculated expected values $E_{ij}(W,Z)$ for each species and then determined the average expected value across all species. Next, we evaluated the model's sensitivity to each variable by determining the proportional change in the average expected value for a unit change in the corresponding variable. We further tested how changes in the variable affected the recommended conservation strategy by varying the values and comparing the number of species for which the conservation strategy would change compared to the original values.

3 Results

3.1 Species representation in EU zoos

In our study, we found that out of the 847 species assessed, EU zoos collectively, held 417 species, representing 49% of all EU species (Species360 Zoological Information Management System (ZIMS), 2021). This corresponds to 53% of birds (253 of 473 spp.), 42% of mammals (70 of 168 spp.), 44% of amphibians (35 of 79 spp.) and 46% of reptiles (59 of 127 spp.). Additionally, EU zoos hosted 39% of all EU threatened or regionally extinct species (60 of 153 spp.), although this proportion varied among taxonomic groups (Figure 2A). Furthermore, they held 27% of species endemic to the EU (47 out of 173 spp.) and 32% of species endemic to Europe (89 out of 279 sp. whose geographic range at least partially included the EU). Analyzing population sizes within EU zoos, we found that mammals had, on average, the largest populations, followed by amphibians, birds, and reptiles. Among threatened species, population sizes ranged from 1 to 1564 individuals (mean = 162, median = 85), with the largest population referring to the European Pond Turtle (*Emys orbicularis*; VU in EU-27). Notably, there were no significant differences in population sizes between threatened and non-threatened species for all four taxonomic groups (Figure 2B, coefficients see Supplementary Table 2).

Regarding vulnerability to climate change, we found that 62% of species considered vulnerable were represented in EU zoos, accounting for 123 out of 197 species (excluding mammals). Specifically, EU zoos hosted 71% of climate-vulnerable birds (101 out of 143 spp.), 42% of reptiles (16 of 38 spp.) and 38% of amphibians (6 of 16 spp.). Additionally, among the five EU species listed by the Alliance for Zero Extinction, EU zoos housed the Montseny brook newt (*Calotriton arnoldi*). Moreover, out of the 49 EU species listed on EDGE, EU zoos kept 12 species (25%).

With respect to *ex-situ* management, a total of 44 EU species (5%) were subject to such conservation efforts, including 19 threatened species. Among these, 21 species (2%) were managed through an EAZA *Ex-situ* Programme, with 12 species managed intensively and nine managed in a European Studbook. Moreover, *ex-situ* conservation efforts were mentioned on the IUCN RL or the Amphibian Ark website for 23 additional species or subspecies (Amphibian Ark, 2019; IUCN, 2019). Notably, while our analysis focuses on species taxa, conservation breeding programs extend to subspecies management, including EAZA's program for the Finnish forest reindeer (*Rangifer tarandus fennicus*) and the Iberian wolf (*Canis lupus signatus*), which is distinct from the separate program for the Grey wolf (*Canis lupus*).

3.2 Prioritization

Recommendations of conservation interventions for the 277 EU species depended on the inclusion of costs in the model. When excluding costs to better capture the effects of benefits and values in the ranking, captive breeding was recommended for 168 (61%) species, habitat protection was recommended for 5 (2%), both interventions for 55 (20%) and no intervention for 49 (18%). When including costs, captive breeding and no action was the preferred intervention for 167 and 50 species, respectively. Habitat protection was recommended for 60 species (22%), and the combined use of both interventions was not recommended (Table 1). To illustrate our findings, we present four examples representing each strategy and taxonomic group (Table 2).

The ranking of species interventions varied depending on the criterion used, such as the inclusion of evolutionary distinctiveness

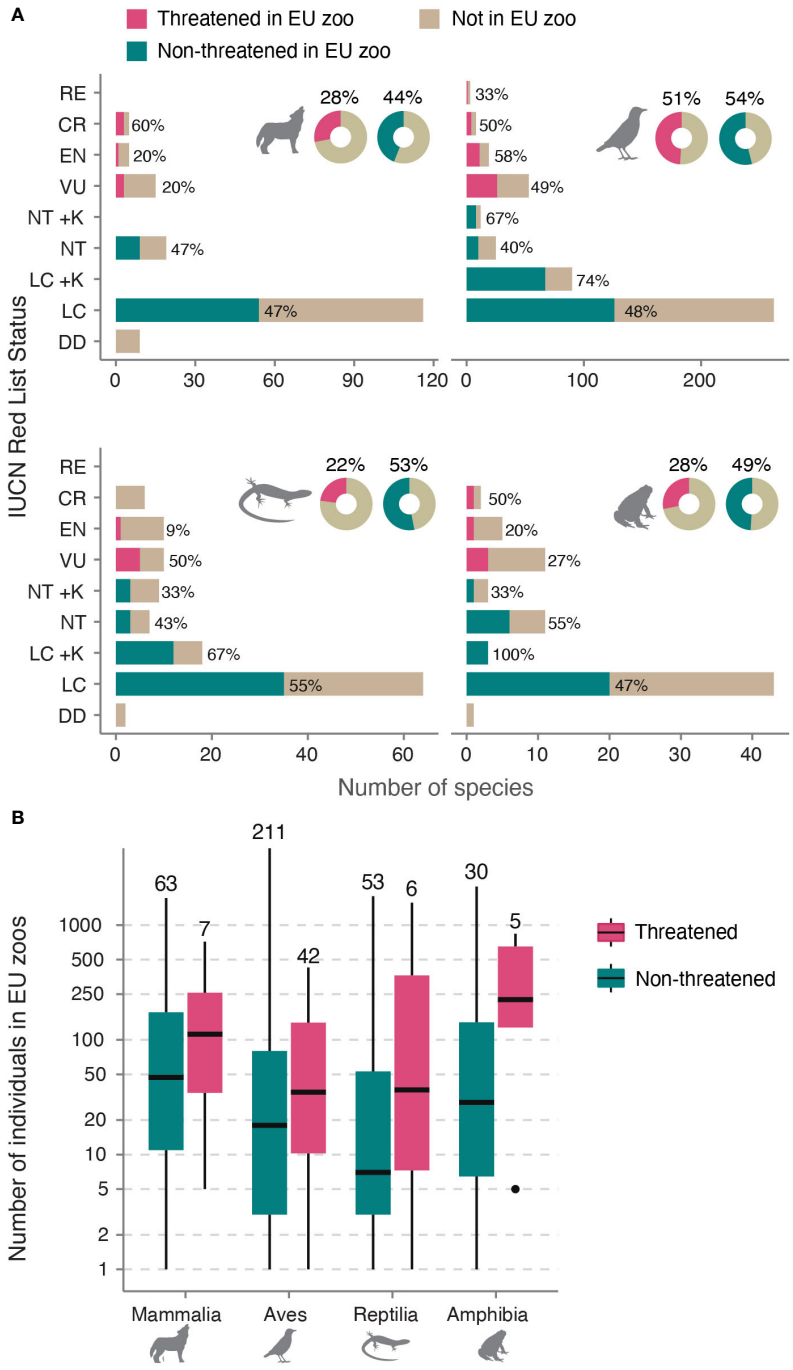

FIGURE 2
Representation of EU species in EU zoos. **(A)** Number of EU species in EU zoos by IUCN status. Near Threatened (NT) and Least Concern (LC) are split into species with high (+K) or low (−K) vulnerability to climate change for birds, reptiles, and amphibians (data not available for mammals). Numbers next to bars indicate the percentage of species per category in EU zoos. Doughnut charts show total percentages. Silhouettes were obtained from www.phylopic.org. **(B)** Boxplots showing the distribution of *ex-situ* holding sizes of threatened vs non-threatened species in EU zoos (on log scale). Values above boxplots show the total sample size in number of species, for example there are 63 non-threatened mammals kept in EU zoos, with a median population size of 49. RE, Regionally Extinct; CR, Critically Endangered; EN, Endangered; VU, Vulnerable; DD, Data Deficient.

and costs (Table 2). For example, in the case of amphibians, the Karpathos frog (*Pelophylax cerigensis*) ranked highest when considering benefits alone, whereas the Mallorcan midwife toad (*Alytes muletensis*) ranked highest when benefits were weighted by evolutionary distinctiveness. When including costs, the Sette Fratelli cave salamander (*Speleomantes sarrabusensis*) was prioritized. Complete data on recommended strategies and species rankings under each strategy can be found in the Supplementary Material.

Comparing our results to the conservation recommendations of the Red List, our model suggests captive breeding as the recommended

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TABLE 1 Number of EU threatened species for which each conservation intervention is recommended.

	Habitat protection (%)	Captive breeding (%)	Both (%)	No action (%)
Net benefits				
Mammals	2	18	5	3
Birds	0	116	11	36
Reptiles	0	23	23	9
Amphibians	3	11	16	1
Total	5 (2)	168 (61)	55 (20)	49 (18)
Cost-effectiveness				
Mammals	7	18	0	3
Birds	11	115	0	37
Reptiles	23	23	0	9
Amphibians	19	11	0	1
Total	60 (22)	167 (60)	0 (0)	50 (18)

Net benefit only captures the expected benefit of an action while cost-effectiveness also incorporates the action's cost in the prioritization depending on the decision-maker's preference.

strategy for 167-168 species, compared to only 11 species on the Red List. Among those 11 species, our model suggested no intervention for 4 species that already have large numbers of individuals in zoos (117–555 individuals). However, these species may nevertheless benefit from coordinated breeding programs.

3.3 Sensitivity analyses

The sensitivity analyses conducted on our model, showed that the most influential factor affecting outcomes is the decision-makers value judgement $V(x,y)$. Particularly important, was the decision-makers valuation of the species persistence in both the wild and captivity ($V(1,1)$) in comparison to persistence in the wild only (Figure 3). Varying this value within a range from its maximum at 1 to 75% of its original value resulted in changes in recommended interventions for up to 73% of species (Supplementary Figure 3). Conversely, we found limited sensitivity to the level of breeding expertise ($P_{i2}(Z,\ G_{ik})$) and the extinction probabilities related to climate change (K_i). Different values of breeding expertise and extinction probabilities pertaining to climate change did not lead to changes in the recommended strategy (Supplementary Figures 1, 2).

4 Discussion

Our findings highlight the considerable potential of EU zoos that share their data in ZIMS for biodiversity conservation within the EU. Currently these zoos house over half (53%) of the 290 EU species that are either threatened or vulnerable to climate change. Yet only ~5% of EU species are currently managed in coordinated *Ex-situ* programs by the EAZA, and population sizes of threatened species were relatively small (median = 85), although not significantly different from those of non-threatened species. In contrast to the current management practices, our model recommended additional investments in *ex-situ* populations for up to 61% of the 277 at-risk species analyzed in the decision analysis. Conversely, expanding habitat protection was recommended for a smaller proportion of species (ranging from 2 to 22% for habitat protection alone, and 20% for both interventions). These results suggest that zoos could significantly enhance their conservation impact by establishing more coordinated breeding efforts for highly prioritized EU species, particularly those already present in EU zoos but facing threats despite adequate habitat protection in the wild. Our framework represents an important first step in supporting zoos and other conservation organizations, whether working individually or collectively, in identifying potential conservation strategies and making informed decisions for captive breeding. By combining extinction risks, values, and costs into a unified metric, our approach can be applied flexibly to a wide range of species, including those with limited available information.

Although our model suggests prioritizing additional investments in captive breeding over habitat protection for many species, it is important to emphasize that preserving a species in its natural habitat remains the primary goal of species conservation. Establishing captive breeding programs for all 167-168 recommended species may not be feasible due to various challenges, such as low breeding success resulting from complex diets (e.g., in bats and seabirds; Conde et al., 2013), limited availability of suitable founding individuals, or limited reintroduction possibilities in the wild. Conservation management strategies must be tailored to each species, considering both captive breeding and habitat protection as parts of an integrated conservation strategy. The ranking of species allows for a targeted focus of highly ranked species first, rather than necessitating the simultaneous prioritization of all species at once.

Long-term captive breeding ultimately leads to loss of fitness of a species due to inbreeding, adaptation to the captive environment, and the loss of species-typical behaviors. However, by managing zoo populations as part of a meta-population with a regular exchange of

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TABLE 2 Prioritization of conservation interventions for four case study species.

Species	IUCN status	Vuln. climate change	PA target met	Nr. in EU zoos	*Ex-situ* expertise	Evol. Distinct.	Recommended strategy		Rank			
							Benefits only	Cost effectiveness	Ri_1 (B)	R_{i2} (B*D)	R_{i3} (B/C)	R_{i4} (B/C)*D
Iberian lynx (*Lynx pardinus*)	CR	NA	100%	14	4	8.4	Captive Breeding	Captive Breeding	7	7	9	9
Azores bullfinch (*Pyrrhula murina*)	VU	High	20%	0	2	4.4	Both	Wild	2	4	6	7
Marginated tortoise (*Testudo marginata*)	LC	High	100%	227	4	24.2	No action	No action	39	47	39	47
Mallorcan midwife toad (*Alytes muletensis*)	VU	High	43%	841	4	36.9	Habitat protection	Habitat protection	7	1	4	2

EU Red List threat status, vulnerability to climate change (K_i), proportion of target protected area met (A_i), number of individuals kept in EU zoos, level of *ex-situ* management expertise (G_i) and evolutionary distinctiveness (D_i) were included in a decision framework to determine whether to implement the following interventions: habitat protection, captive breeding, both, or none of these actions based on either net benefit (B_i) or cost-effectiveness (B_i/C_i). Species rankings when projects are prioritized based on benefits only (R_{i1}), benefits weighted by evolutionary distinctiveness (R_{i2}), cost-effectiveness (R_{i3}) or weighted cost-effectiveness (R_{i4}). NA, Not Available. Full species rankings are provided in the Supplementary Material.

breeding stock between *in-situ* and *ex-situ* populations, the negative impacts can be minimized, and the effective population size can be increased (Lacy, 2013). Given data constraints, our determination of the minimal viable population size relied on total population counts rather than effective population size and did not consider the population's genetic diversity. Notably, our current model needs further refinement to incorporate more nuanced demographic and genetic models that account for these additional factors. Moreover, the threshold of 100 individuals as a viable population size has been widely debated, with suggested numbers varying between 50 and 5000 individuals (Lacy, 2013). The specific number will vary depending on species-specific traits, such as generation time, reproductive strategy, and species management. Large population sizes may not always be necessary. Notably, some species have demonstrated the ability to recover from extremely low population sizes, while others may have naturally existed with low genetic diversity for millennia (e.g., on islands) (Wiedenfeld et al., 2021).

In terms of habitat protection, we adopted the targets defined by Maiorano et al. (2015). Most species in our analysis (217 out of 277) already met those targets, resulting in a lower number of fewer species recommended for additional investments in habitat protection. However, despite meeting these targets, species continue to face increasing threats in the wild. While habitat loss and climate change are recognized as primary threats to many EU species, their impact on extinction risk remains poorly understood. Species survival will depend on various other factors, such as the effectiveness of management efforts in protected areas (Geldmann et al., 2013) control of invasive species, human disturbances, and disease management (European Commission, 2020).

Our prioritization relies on European IUCN Red List assessments, which restricts the scope of this study to species recognized by this framework. Insufficient taxonomic research, for example for European mammals, greatly impacts conservation policies in Europe, often neglecting subspecies due to data gaps and changing taxonomic lists. For example, out of the 24 mammals occurring in Italy, for which species status has been proposed since 2005, only one (*Neomys milleri*) is currently recognized by the IUCN Red List and has been assessed globally, but not regionally (Gippoliti and Groves, 2018; Gazzard and Meinig, 2023). Recognizing that taxonomic research is the cornerstone for effective conservation strategies, it becomes evident that enhancing taxonomic efforts as well as supporting efforts to assess these species within the IUCN regional frameworks is imperative. Moreover, additional research is needed to refine extinction probabilities, taking into account climate-related threats. Here, to develop probabilistic analyses, we transformed categorical values (IUCN RL categories and climate change vulnerability) into probabilities. We assumed that species categorized as vulnerable to climate change would experience increased risk of extinction and that protecting a species' target area would linearly decrease extinction risk and have an equal impact on all species. However, the extent to which climate change affects extinction risk on a per-species basis, and how this translates to probabilities, remains uncertain.

Despite these limitations, our model, which includes species not currently classified as threatened but facing future threats (such as climate change), presents a proactive conservation approach compared

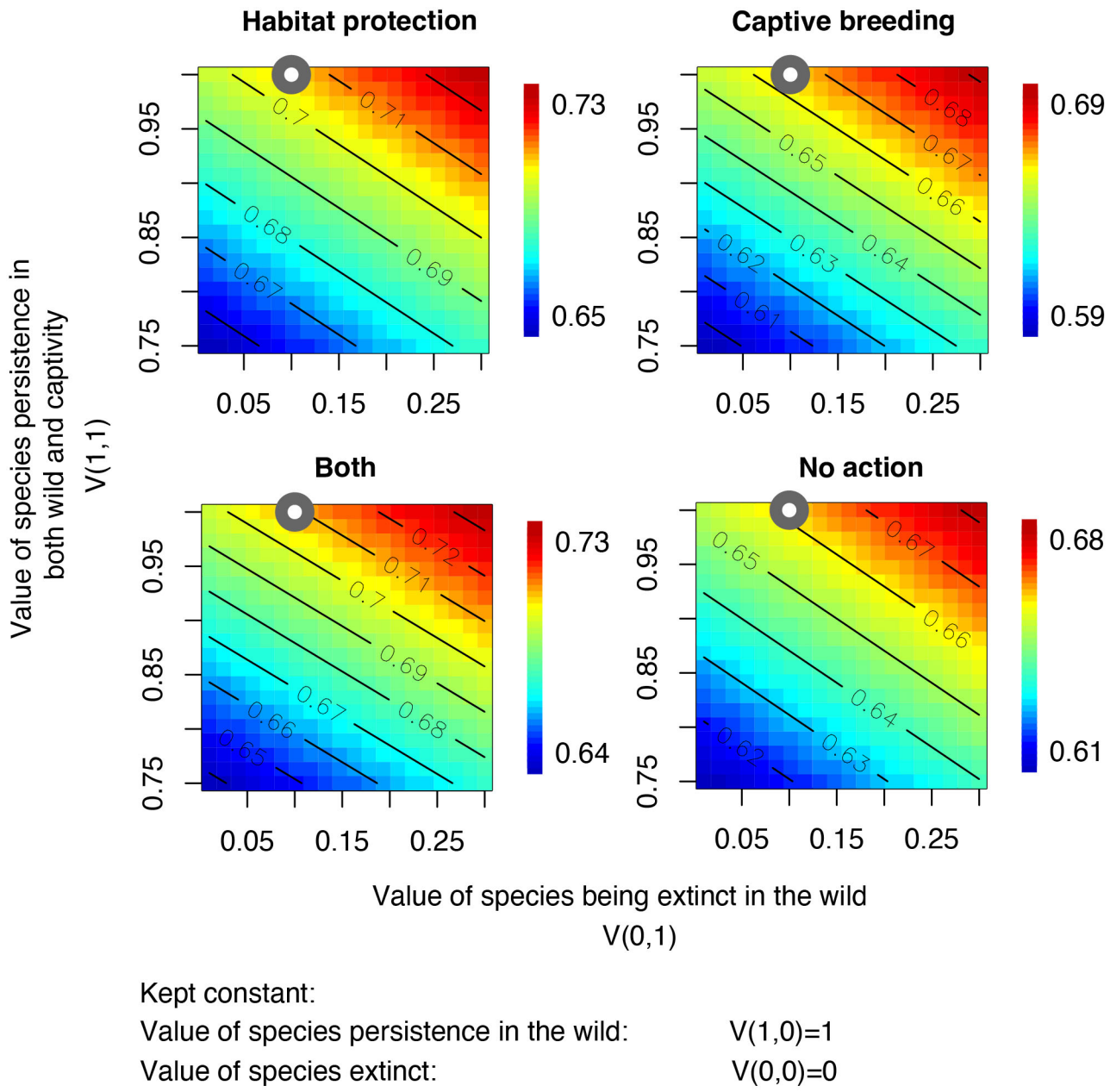

FIGURE 3
Sensitivity analysis of the effects of value judgments on expected values of the four conservation interventions. The x-axes show variations in the judgment value *V*(0,1) (i.e., the value of a species being extinct in the wild ranging from 0 to 0.3) and the y-axes show variations in the judgment value *V*(1,1) (i.e., value of species persistence in both the wild and captivity, ranging from 0.8 to 1). The values of a species persisting in the wild (*V*(1,0) = 1) and becoming extinct (*V*(0,0) = 0) were kept fixed. Color scales show mean expected values. Grey points indicate the values selected in the analysis.

to the reactive approach of extracting individuals from already small populations. For example, the common hamster (*Cricetus cricetus*) has recently been uplisted from Least Concern to Critically Endangered on the global IUCN Red List, despite being a previously abundant species in Europe and Russia (Banaszek et al., 2020). Presently, there are approximately 481 individuals of this species in EU zoos registered in ZIMS (Species360 Zoological Information Management System (ZIMS), 2021), which can be managed to establish insurance populations until threats in the wild are reduced, if at all. Furthermore, our model allows for the testing of assumptions and enables further refinement of prioritization approaches as more comprehensive and improved data become available. For example, we found that varying estimates of captive breeding expertise and extinction risk from climate change had minimal impact on our results, suggesting these variables influenced decisions and rankings less than estimates of costs or value judgments by decision-makers.

A crucial aspect that requires improvement upon is the estimation of captive-breeding costs, as accurate data are currently unavailable. When deciding between alternative options, most individuals will naturally take costs into consideration, and a prioritization

framework aims to make these costs explicit. We emphasize the importance of including costs in the analysis, as neglecting them could result in misallocation of resources. Investing heavily in saving highly threatened species with limited potential for recovery while disregarding lesser threatened species that require fewer resources may lead to suboptimal outcomes (Joseph et al., 2009). However, resources may not be as limited as commonly assumed. For example, Wiedenfeld et al. (2021) argue that additional funding could be leveraged from previously unexplored sources, such as the private sector.

To accommodate the different perspectives, we present two approaches: one including and one excluding costs. The cost-effectiveness analysis reveals that the additional contribution to species conservation from captive breeding incurs much higher expenses compared to habitat protection (~1 billion € for captive breeding versus a total cost of ca. 5 million € for habitat protection if all species are protected). However, the costs of habitat protection are likely underestimated, as they are based on average country-level costs and do not consider the specific distribution of the species. Similarly, the cost of captive breeding, particularly for birds and mammals, is solely based on body mass. Consequently, we urge zoos to make information regarding costs of keeping and managing their animals available to facilitate more informed decision-making.

The goal is to prevent species extinction in the wild and, ultimately, the complete loss of a species. Thus, our model assigns lower value to the outcome that a species only persists in zoos, while placing higher value on the outcomes of the species persisting in the wild or in both zoo and wild settings. The recommendations were highly sensitive to those hypothetical decision-makers' preferences. Consequently, if our model is utilized to select among various options, it is crucial to carefully elicit and consider the preferences of the decision-makers involved (Rout et al., 2013). The societal view on captive breeding programs in Europe, and how to evaluate the significance of species becoming extinct in the wild will be crucial in shaping discussions about species conservation strategies in the future.

While our study relies on generalizations among species, it serves as a valuable initial step towards prioritizing species for the rigorous assessment process outlined in the IUCN *Ex-situ* guidelines. While our framework primarily focuses on additional investments to current conservation efforts in habitat protection and captive breeding, it has the potential to be expanded to include other crucial conservation actions. These actions may include temporary rescue efforts, establishing sources for reintroduction, population restoration or assisted colonization initiatives, research, training, prioritization of genetically important individuals for a species, and the collection of frozen live cells for genetic rescue (IUCN/SSC, 2014). Further evaluation against the IUCN *Ex-situ* guidelines will capture additional factors not incorporated into our analysis, such as country-level regulations on pollutants that directly impact a species, human population pressure, and the genetic variability. As in other data-intensive assessment schemes (such as IUCN Red List extinction risk assessments), initial screening and prioritization of species may be useful (Bland et al., 2015).

Given the current extinction crisis, EU zoos are uniquely positioned to prevent further extinctions of EU threatened species but will need to orient themselves to managing populations more sustainably. Our framework can facilitate further critical analysis to maximize the impact of *ex-situ* actions to support species survival in the wild and align zoo conservation efforts with existing EU Biodiversity targets.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. R code is available on GitHub at: https://github.com/jostaerk/Staerk_etal_EU_Zoos. Further inquiries can be directed to the corresponding authors.

Author contributions

JS: Data curation, Formal Analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. FC: Methodology, Visualization, Writing – review & editing, Funding aquisition. MK: Writing – review & editing, Validation. KW: Data curation, Writing – review & editing. WF: Data curation, Writing – review & editing. JC: Data curation, Writing – review & editing. ZP: Writing – review & editing. LB: Writing – review & editing. NF: Writing – review & editing. TM: Writing – review & editing. LM: Data curation, Writing – review & editing. JF: Writing – review & editing. HP: Methodology, Writing – review & editing. DC: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author LB was employed by company Eurika Publishing.

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

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The influence of pair duration on reproductive success in the monogamous 'Alalā (Hawaiian crow, *Corvus hawaiiensis*)

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Conservation breeding program practitioners select potential mates in an attempt to maximize pair compatibility and maintain genetic diversity. Therefore, pair duration, or the number of breeding seasons that individuals retain the same mate, is practitioner-determined in these settings. There is a critical need to evaluate whether pair duration influences reproductive success in *ex situ* assurance populations, particularly for socially monogamous species. The 'Alalā (Hawaiian crow, *Corvus hawaiiensis*) is a monogamous forest bird that is currently extinct in the wild. Today, 'Alalā exist only in human care for intensive conservation breeding. We analyzed breeding program data from 2018-2021 to determine the effects of 'Alalā pair duration and age on reproduction (nest building, egg laying, hatching, and fledging). We found that pair duration does not influence reproductive outcomes, and thus practitioners can be more proactive when re-pairing birds. Female and male age, on the other hand, influenced the probability of nest building, clutch production, and overall reproductive success. Nest building and clutch production probabilities were high (near 1) and stable as females aged from 2 to ~ 12 years old, declining sharply thereafter. In males, overall reproductive success (from building robust nests to rearing at least one nestling to fledge) increased with age from 2 to ~ 9 years old, peaked and reached an asymptote with males $\gtrsim$ 9 to ~ 13 years old, and decreased in males $\gtrsim$ 13 years old. Thus, integrating age into the pair selection process will increase the likelihood of achieving conservation goals. To our knowledge, we are the first to utilize empirical pair duration results to provide specific management recommendations for mate selection in an avian conservation breeding program. Our findings have critical utility for guiding 'Alalā pairing decisions, and more broadly underscore the importance of evaluating mate retention and selection protocols in other conservation breeding programs.

KEYWORDS

mate compatibility, extinct in the wild, mate selection, pair bond, mate retention, aviculture

Introduction

Conservation breeding is an important tool for animal conservation used to save species from extinction and achieve species recovery goals by establishing *ex situ* assurance populations often for reintroduction and translocation. Breeding practitioners are inherently faced with limited resources and small sample sizes (in terms of the total number of individuals available to breed), as well as sparse data on the species prior to being brought into human care which are typically collected during a time when wild populations were already in marked decline. Thus, practitioners must make husbandry decisions while being immersed in uncertainty. Yet, with an adaptive management framework, conservation breeding practitioners can continually adjust and refine decisions as new information becomes available. Specifically, by recording and analyzing detailed data on reproductive outcomes, as well as their potential drivers, practitioners can take a scientific approach to guide and adapt management decisions for their unique programs (Heinrichs et al., 2019).

A major challenge in conservation breeding programs is identifying potential mates that will successfully produce offspring. Mate selection can involve a "hands-off" approach by providing animals with the opportunity to choose their own mate from a pool of contenders (e.g., in mate choice studies; Ihle et al., 2015; Martin-Wintle et al., 2019; Munson et al., 2020). Other approaches may involve pairing individuals based on criteria such as whether a pair would positively contribute to the genetic health of the population, assuming offspring are produced (e.g., by minimizing inbreeding and retaining founder representation; Montgomery et al., 1997; Ballou et al., 2010; Ivy and Lacy, 2012). Alternatively pairs may be chosen based on assessments of behavioral compatibility (e.g., based on personality type; Smith and Blumstein, 2008; Martin-Wintle et al., 2017; Faust and Goldstein, 2021), or a hybrid approach that considers both genetic and behavioral compatibility. For species that cannot be housed in a communal setting, practitioners must make difficult decisions about when to divorce and re-pair previously selected mates, which becomes necessary, particularly if the pair has been repeatedly unsuccessful at breeding. When faced with these decisions, practitioners can draw knowledge from the animal's life history, such as its mating system. When working with a monogamous species with strong mate fidelity, long-term mate retention may be desirable. In contrast, for species that are not monogamous or have part-time partnerships (Black, 1996), practitioners may re-pair potential breeders more frequently or allow individuals to have simultaneous access to multiple potential mates. Despite the mating system of the species, in the context of conservation breeding, it is vital to determine if, when, and how often to re-pair animals to maximize productivity. Critical to this decision is assessing the effects of mate retention (referred to as pair duration in this study). However, the effects of pair duration in conservation breeding programs (in monogamous species) have not been thoroughly studied, particularly in birds.

Previous work across numerous taxa of socially monogamous species with strong mate fidelity, predominantly in the wild, has shown that individuals that retain the same mate for longer periods of time have higher reproductive success. There are many probable mechanisms explaining the positive relationship, or association, between pair duration and better reproductive outcomes (e.g., resource-based, reproductive performance, and mate familiarity hypotheses underpinning pair fidelity, reviewed in Leu et al., 2015). While the resource-based hypothesis may be irrelevant to birds in *ex situ* breeding programs, as individuals in these settings do not face the same resource limitations as their wild counterparts, the reproductive performance and mate familiarity hypotheses may apply. The reproductive performance hypothesis predicts that older and more experienced pairs are more likely to achieve reproductive success than younger, inexperienced pairs, and thus mate retention is preferable to divorce (i.e., splitting from an established mate to seek another) in the context of reproductive success (Leu et al., 2015). The mate familiarity hypothesis predicts better reproductive outcomes in pairs that retain the same mates because familiarity with one another enables pairs to breed more efficiently with coordinated reproductive behaviors (Leu et al., 2015). For instance, in Australian sleepy lizards (*Tiliqua rugosa*), pairs in which mates were more familiar with one another tended to breed earlier than unfamiliar pairs (Leu et al., 2015). Likewise, gray wolves (*Canis lupus*) that kept the same mate for longer periods of time had higher apparent offspring survival (Ausband, 2019). Other work has suggested that birds with more familiar or compatible mates better coordinate incubation and provisioning of young (Spoon et al., 2006 but see Ihle et al., 2019 who found that coordination of mates did not improve offspring condition or survival). Moreover, experimental work with bearded reedlings (*Panurus biarmicus*) found that pairs that were together longer were better coordinated, bred earlier, and had higher hatching and fledgling success than newly formed pairs (Griggio and Hoi, 2011). This pattern has also emerged in peregrine falcons (*Falco peregrinus*) living in human care, where longer-term mates produced more fledglings during their lifetime than birds that did not retain their mates (Clum, 1995).

Alternatively, some individuals experience benefits to seeking a new mate instead of retaining their original mate (e.g., better option hypothesis, Ens et al., 1993). For example, in cockatiels (*Nymphicus hollandicus*), pair duration did not correlate with the number of eggs or nestlings produced, providing evidence against the mate familiarity hypothesis (Spoon et al., 2016). Other work has shown that, in some cases, it is beneficial for animals to acquire new mates after a successful breeding season (Kelley et al., 1999). In plovers (*Charadrius* spp.), for example, divorced birds produced more hatchlings than birds that retained mates, and divorce was more likely when a nest hatched successfully (Halimubieke et al., 2020). This idea has also been supported in island foxes (*Urocyon littoralis*) that were part of an *ex situ* breeding program, as well; newer pairs had a higher probability of reproductive success compared to more established pairs, a result that has important implications for unsuccessful pairs in conservation breeding programs in terms of mate selection (Calkins et al., 2013). Given these discrepant results,

it remains unclear how pair duration affects reproductive success for bird species in human care.

The ʻAlalā (Hawaiian crow; *Corvus hawaiiensis*) (Figure 1), the only remaining endemic corvid species found in Hawai'i, is presently extinct in the wild. Attempted reintroductions that occurred in the 1990s (Kuehler et al., 1995) and from 2016-2020 (Smetzer et al., 2021) did not produce any self-sustaining wild populations. Thus, at the time of this writing, all living individuals reside in human care for intensive conservation breeding. Here, we tested whether pair duration influenced ʻAlalā reproductive success using detailed data on reproductive outcomes from 2018-2021 (Figure 1). After a sufficiently large assurance population was established, from ~ 2018 onward, (with ~ 140 living individuals), the breeding program moved away from intensive, traditional avicultural methods (involving artificial incubation and puppet-rearing offspring) to parental breeding with pairs being predominantly full-time socialized to allow for coordinated breeding behaviors to occur. The transition to parental breeding was an important shift in management intended to encourage the birds to successfully build nests, incubate eggs, and parent-rear nestlings, with important implications for animal welfare and, eventually, reintroduction to the wild (Flanagan et al., 2023).

When ʻAlalā were observed in the wild, they were observed in monogamous pairs with strong mate fidelity (Banko et al., 2002). Based on this and work with other monogamous species (e.g., Clum, 1995; Spoon et al., 2007; Griggio and Hoi, 2011; Ihle et al., 2015), we assumed that pair duration, albeit artificially imposed, would be an adequate proxy for pair compatibility, due to the fact that incompatible pairs are separated and re-paired. We therefore predicted that pair duration would be positively related to reproductive success, due to better compatibility and/or greater familiarity, where mates that were together longer would have a greater probability of engaging in nest building, producing a clutch, and achieving other downstream reproductive milestones, such as rearing nestlings to fledge. While this study design does not allow these underlying mechanisms to be fully understood, it does allow us to evaluate alternate management strategies related to maintaining pairs. Testing this hypothesis will inform practitioners' decisions about maintaining existing pairs versus separating and re-pairing with different individuals across reproductive seasons. To our knowledge, we are the first to leverage pair duration data from an avian conservation breeding program to produce results with direct links to management recommendations for mate selection.

FIGURE 1
An ʻAlalā pair at Maui Bird Conservation Center. Photo credit: Mālie Naho'olewa.

Methods

Summary of the conservation breeding program

ʻAlalā conservation breeding is conducted at two locations in Hawai'i: the Maui Bird Conservation Center (MBCC) on Maui and the Keauhou Bird Conservation Center (KBCC) on Hawai'i Island. The breeding season starts in April and ends in August of each year. Potential mates are selected prior to the start of the breeding season on an annual basis, using genetic and demographic criteria, along with caretaker-perceived behavioral compatibility, and, more recently, mate choice (Greggor et al., 2018) and personality studies. Throughout our study, established successful mates, particularly those that have produced offspring, were kept together, as the ʻAlalā is long-lived (some individuals live for up to ~ 30 years) and was observed to have strong mate fidelity in the wild (Banko et al., 2002). Regardless of reproductive success, or lack thereof, most pairs in our study were kept together for more than one breeding season (71%), based on the rationale that some pairs may need to be together for multiple seasons prior to reaching various reproductive milestones (nest building, egg laying and incubation, hatching eggs, and rearing chicks to fledge). Some first year pairs were split up and re-paired, particularly if there were clear behavioral indicators of incompatibility observed, such as observations of highly concerning or persistent aggression (e.g., fighting), which can compromise the safety of the birds. However, it is generally unclear whether unsuccessful mates should be separated to find more suitable mates in an effort to improve breeding outcomes. All pairs reside in open-air aviaries with wire-mesh walls, and most aviaries house a single pair. Throughout our study, pairs remained socialized except when caretakers occasionally moved one member of a pair into a separate, but adjacent aviary compartment (within the same building) to administer medication to a sick bird or to address pair compatibility issues, such as severe or persistent aggression.

Reproductive data recorded

ʻAlalā commonly lay 2-3 eggs per clutch, producing 2-3 clutches per breeding season, unless the pair is successful at hatching and rearing nestling(s) from their first clutch. Caretakers monitored all breeding activities in-person during daily husbandry and by closed-circuit television. ʻAlalā pairs were provided with 2-5 nest building platforms (Figure 2) in addition to an assortment of nest materials such as sticks, grasses, and coconut fibers. Throughout our study, caretakers recorded

FIGURE 2
Examples of the nest building platforms offered to ‘Alalā. Dimensions vary among platform designs, with maximum widths ranging from 0.4-1.1 m.

nest progress data to capture whether each pair had placed no sticks, a few sticks, many sticks, or constructed a nest with a visible nesting cup, on one or more nest platforms in their aviary, at least three times per week, beginning March 1st, until females laid their first clutch. Nests with eggs were assigned a discrete, ordinal nest quality score, ranging from 1 (worst nest; essentially no attempt at nest building) to 5 (best nest) at the time of lay (Flanagan et al., 2023). In addition to collecting data on nest progress and nest quality, caretakers monitored and recorded data on egg laying, hatching, and nestling survival. Because the conservation breeding management approach taken throughout our study was centered on parental breeding, we did not examine most eggs for signs of fertility to minimize human disturbance.

Reproductive outcomes evaluated

We tested whether pair duration predicted nest building, production of one or more clutches, and overall reproductive success (in pairs with a minimum of one clutch). We examined potential pair duration effects on nest building to capture evidence of breeding behaviors, or lack thereof, particularly in pairs that did not necessarily produce clutches (but pairs with clutches were also included in this analysis). We operationalized nest building attempts as nests containing a minimum of “many sticks.” Although we currently do not have sufficient data to formally analyze differences in reproductive outcomes associated with the various nest platform types provided, preliminary assessments suggest that nest building behaviors do not vary systematically with platform type. Because not all females consistently lay from year-to-year, we also investigated potential pair duration effects on the probability of clutch production. Overall reproductive success was measured by assigning each pair a discrete, ordinal “success” score ranging from 0-3: 0 = pair laid a clutch of eggs in a low-quality nest (scored < 4), 1 = pair laid a clutch of eggs in a high-quality nest (scored ≥ 4), 2 = pair had ≥ 1 hatchling, and 3 = pair had ≥ 1 fledgling (at ~ 60 days after hatch).

Statistical analyses

All of our analyses were conducted in R Studio (R Core Development Team, 2023). We constructed global models for the nest building (n = 161 observations, 75 dyads, and 4 breeding seasons), clutch production (n = 164 observations, 75 dyads, and 4 breeding seasons), and overall reproductive success analyses (n = 168 observations, 52 dyads, and 4 breeding seasons). All global models included breeding season (year) and dyad (pair identity) as random effects. We ran separate models with pair duration as a numeric fixed effect (number of years paired) and as a binary fixed effect (i.e., whether a pair had ≥ 1 prior breeding season together), the latter of which was intended to capture if simply having experience together as a pair impacts reproductive outcomes (vs. the temporal extent of experience or years paired). To incorporate potential age effects, we included the age of the birds and age^2, based on the assumption that reproductive outcomes may vary nonlinearly with age, in addition to age × pair duration interaction terms. In addition to the age covariates, we included clutch number in the model of overall reproductive success to account for the possibility that earlier/later clutches may be associated with varying levels of reproductive success (e.g., in terms of hatching or nestling survival to fledge). All fixed effects were standardized with the arm package (Gelman and Su, 2018), and multicollinearity was evaluated with variance inflation factors (VIF), calculated in the car package (Fox and Weisberg, 2011). We used binomial generalized linear mixed models (GLMMs) for the analyses of nest building and clutch production, both fitted with a logit link function. As the reproductive success scores were ordinal, we used a cumulative linked mixed model (CLMM) for this analysis. We checked model assumptions with the DHARMa

(Hartig, 2022) and ordinal (Christensen, 2018) packages for the nest building and clutch production GLMMs and the reproductive success CLMM, respectively.

The set of submodels utilized in model averaging were derived from the global models using the dredge function in the MuMIn package (Barton, 2018). Model averaging included all submodels within 2 AICc of the most parsimonious model (i.e., the model with the lowest AICc score), and was conducted with the natural average method. We used the relative importance (RI) scores generated from model averaging to guide inferences made from the results, which we limited to fixed effects with high RI scores ($\gtrsim$ 0.8).

Results

ʻAlalā pair duration ranged from 0 to 10 consecutive breeding seasons across the dyads included in our study (2.5 ± 0.2 SE). Males and females in our study were 2-19 (9.6 ± 0.3 SE) and 2-20 (8.9 ± 0.3 SE) years old, respectively. We removed year as a random effect from the global model of nest building because near 0 variance was associated with this term, causing model singularity. Male age × pair duration was removed from our clutch production analysis (with pair duration as a numeric effect), as this interaction term had VIF > 5; however, we were able to retain these interactions in the model with pair duration as a binary fixed effect (all fixed effects had VIF < 5 in this analysis).

We did not find any relationships between pair duration and nest building, clutch production, or overall reproductive success (Tables 1–3), regardless of whether pair duration was treated as a numeric (0-10 years) or binary (pair had some or no experience together in consecutive breeding seasons) fixed effect. As such, the results presented here are from the models that utilized pair duration as a numeric effect, but the results from models with a binary structure for pair duration are provided in the Supplementary Material, in addition to all submodels used in model averaging.

Although we did not detect relationships between pair duration and reproductive outcomes, we found some evidence suggesting female age impacted the probability of nest building and laying ≥ 1 clutch (Tables 1, 2; Figure 3). Specifically, the probability of nest building and clutch production was relatively high (near 1) and stable as females aged from 2 to ~ 12 years old, declining sharply thereafter. For pairs with a minimum of one clutch, we found that male age had an important effect on overall reproductive success (Table 3; Figure 4). Reproductive success scores increased as males aged from 2 to ~ 9 years old (across all ordered success score categories), reached a peak and an asymptote with males $\gtrsim$ 9 to ~ 13 years old, and subsequently decreased in males $\gtrsim$ 13 years old (Figure 4).

Discussion

We tested whether pair duration in the critically endangered ʻAlalā influenced reproductive outcomes across four breeding seasons during which a parental breeding management approach was adopted for the species. Our results clearly show that pair duration did not impact the probability of nest building, clutch production, or downstream reproductive outcomes, including the successful rearing of nestlings to fledge. Although senescence was not the focus of our study, we found that female age influenced the probability of nest building and clutch production, and male age affected overall reproductive success.

TABLE 1 Model-averaged GLMM results for nest building attempts.

Parameter	β	SE	95% CI	VIF	RI
Intercept	2.6976	0.6271	(1.47, 3.93)	–	–
Female age	-1.1100	0.7628	(-2.61, 0.39)	3.3910	0.35
Female age^2	-1.6486	0.8232	(-3.26, -0.04)	2.7652	0.81
Male age	-1.0099	0.6335	(-2.25, 0.23)	2.9526	0.40

Parameter estimates, uncertainty (standard errors and 95% confidence intervals), Variance Inflation Factors (VIF), and Relative Importance (RI) scores are reported for each fixed effect.

TABLE 2 Model-averaged GLMM results for clutch production.

Parameter	β	SE	95% CI	VIF	RI
Intercept	4.2826	2.1644	(0.04, 8.52)	–	–
Duration	-0.5818	1.1811	(-2.9, 1.73)	1.5934	0.14
Female age	-2.3263	1.3921	(-5.05, 0.4)	2.0858	0.27
Female age^2	-4.7353	2.8118	(-10.25, 0.78)	2.2992	0.86
Male age	-3.2469	1.9778	(-7.12, 0.63)	2.1237	0.73
Male age^2	-2.7810	1.8491	(-6.41, 0.84)	1.5390	0.51

Parameter estimates, uncertainty (standard errors and 95% confidence intervals), Variance Inflation Factors (VIF), and Relative Importance (RI) scores are reported for each fixed effect.

TABLE 3 Model-averaged CLMM results for reproductive success scores.

Parameter	β	SE	95% CI	VIF	RI
Transition threshold 0|1	-0.6452	0.4053	(-1.44, 0.15)	–	–
Transition threshold 1|2	2.1026	0.4461	(1.23, 2.98)	–	–
Transition threshold 2|3	3.4100	0.5241	(2.38, 4.44)	–	–
Clutch	-0.2114	0.3809	(-0.96, 0.54)	3.79	0.10
Duration	-0.9610	0.6331	(-2.2, 0.28)	1.02	0.38
Duration * Male age	1.4050	1.1865	(-0.92, 3.73)	1.98	0.15
Female age	-0.4763	0.6342	(-1.72, 0.77)	2.18	0.11
Male age	1.3043	0.7218	(-0.11, 2.72)	3.18	0.85
Male age^2	-1.8194	0.8142	(-3.42, -0.22)	1.91	1.00

Transition thresholds (i.e., model-averaged intercepts) are the cumulative probabilities that reproductive success scores fall within or below each discrete score (0-3) on a logit scale. Parameter estimates, uncertainty (standard errors and 95% confidence intervals), Variance Inflation Factors (VIF), and Relative Importance (RI) scores are reported for each fixed effect. The ordinal response levels were: 0 = pair laid clutch in low quality nest (scored < 4), 1 = pair laid clutch in high quality nest (scored ≥ 4), 2 = pair had ≥ 1 hatchling, and 3 = pair had ≥ 1 fledgling.

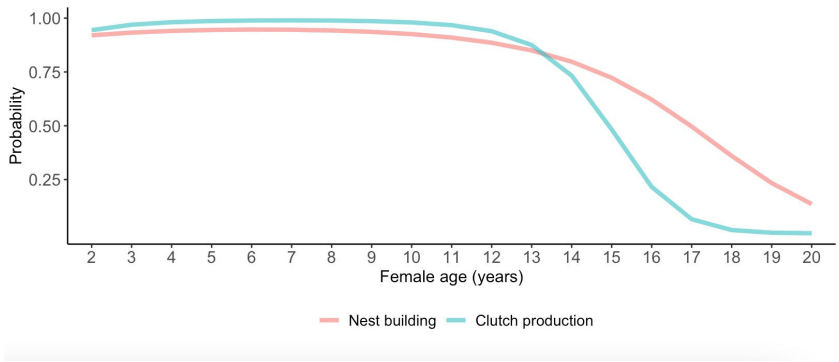

FIGURE 3
Female age effects on the probability of laying ≥ 1 clutch. Predictions in this figure were calculated using the model-averaged intercept and slopes of age and age^2 to illustrate quadratic age effects, and back-transformed using the invlogit function to facilitate interpretability (Gelman and Su, 2018). The probability estimates were calculated with all other parameters in the model being at their means.

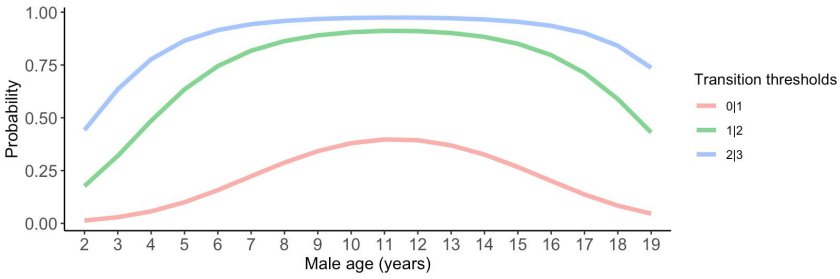

FIGURE 4
Male age effects on reproductive success scores. Predictions in this figure were calculated using the model-averaged intercept and slopes of age and age^2 to illustrate quadratic age effects, and back-transformed using the invlogit function to facilitate interpretability (Gelman and Su, 2018). Ordinal response levels include: 0 = pair produced a clutch in a low-quality nest (scored < 4), 1 = pair produced a clutch in a high-quality nest (scored ≥ 4), 2 = pair had ≥ 1 hatchling, and 3 = pair had ≥ 1 fledgling. The probability estimates were calculated with all other parameters in the model held at their means.

Our results suggesting that pair duration is a poor predictor of reproductive outcomes are, at first glance, somewhat surprising given that the ʻAlalā is a monogamous species, with a history of lifetime pair bonds in the wild (Banko et al., 2002). However, it is important to keep in mind that all ʻAlalā pairs in the breeding program were practitioner-selected. Moreover, detecting pair duration effects on reproductive outcomes may be difficult given the dataset analyzed. For example, pair duration was not an experimental treatment assigned randomly to ʻAlalā pairs, so our conclusions may be confounded by other factors related to caretaker decisions to split pairs. There are two fundamental reasons why caretakers may decide to split a pair: behavioral signs of incompatibility such as aggression and failure to demonstrate reproductive behavior or output. To the extent that a bias exists in these decisions, the bias would support leaving pairs together for longer periods of time if they have greater success. It is difficult to disentangle cause and effect, as pairs may be left together for longer periods of time because they are reproductively successful, or pairs may be reproductively successful because they are left together for longer periods of time. However, this potential bias actually makes our inferences about the management decisions more robust, as the bias should skew the data in favor of higher reproductive success for pairs kept together for longer periods. Yet, our results do not support this hypothesis and in fact we found no relationship between pair duration and reproductive success despite having the odds stacked in favor of higher reproductive success with longer pair duration. Our main conclusion that there is little to be gained from leaving unsuccessful pairs together is therefore more strongly supported in the face of this bias from confounding variables. Moreover, pair duration may not translate well as a "pair bond" and may therefore provide an inaccurate measure of mate compatibility, nor does it capture the biological effects derived from familiarity. In the context of conservation breeding, familiarity may be unimportant, or masked by, the effects of incompatibility, particularly if many pairs in the flock simply tolerate one another but are not motivated to mate, incubate, and/or rear chicks as a pair. Ongoing work on mate choice and compatibility in this population will provide valuable information on improving the compatibility of pairs in the flock (Greggor et al., unpublished data). Another extension of this work could involve testing whether the amount of time that has passed since an individual has been separated from its partner, without immediate re-pairing, influences its ability to form a strong preference and/or stable bond with a new mate (e.g., Harbert et al., 2020). However, all females in our care are immediately re-paired after any mate separations, so this test would only be relevant to the males in our population (i.e., because there are more males than females in the flock).

Although age was not the focus of this study, we detected relationships between age and reproductive outcomes. The probabilities of nest building and clutch production were similar in pairs with females from 2 to 12 years old. However, nest building and clutch production probabilities notably declined after females reached ~ 13 years old. Moreover, male age influenced overall reproductive success in pairs with a minimum of one clutch: pairs with males aged $\gtrsim$ 9 to ~ 13 years attained higher reproductive success scores compared to pairs with males from 2 to ~ 8 years old and pairs with older males, $\gtrsim$ 13 years old. Our findings therefore suggest that female senescence impacts the earlier stages of reproduction, and male age has a role in influencing nest quality and downstream reproductive success, including hatching and nestling survival to fledge, perhaps due to their role in assisting their mates with nest building, feeding females at the nest during the egg incubation and rearing stages, and cooperatively rearing nestlings. There is support for these findings demonstrating the importance of age in the literature. Recent work with monogamous mountain chickadees (*Poecile gambeli*) showed that age or an individual's breeding experience (vs. pair duration) affected parental investment; more experienced pairs produced eggs earlier and raised heavier chicks compared to inexperienced pairs (Pitera et al., 2021). This finding was largely driven by female age/experience, since experienced females initiated egg laying earlier and laid larger clutches than inexperienced breeding females (Pitera et al., 2021). Similarly, reproductive performance of brown thornbills (*Acanthiza pusilla*) improved with age but not with repeated breeding attempts with the same partner (Green, 2001). Researchers have proposed several restraint hypotheses (cost of reproduction, residual reproductive value hypotheses) and constraint hypotheses (selection, breeding experience, and breeding age hypotheses) to explain the pattern that performance improves with age until "middle age" (reviewed in Robertson and Rendell, 2001). For example, it is possible that birds acquire critical, non-breeding-related skills as they age (until they reach senescence or otherwise reach older age with reduced breeding experience), such as self-maintenance or foraging (Robertson and Rendell, 2001). However, given that the birds in our study do not need to compete for food with conspecifics (aside from with their mate), this is an unlikely scenario (Griggio and Hoi, 2011). Alternatively, or additionally, younger females could balance the costs of current reproduction against the probability of future reproduction, resulting in lower reproductive success when they are younger (reviewed in Fowler, 1995). Moreover, in our study, after age 7-12 for females and $\gtrsim$ 13 for males, reproductive success declined; "middle age" reproductive success decline has been well-documented in other bird species (e.g., short-tailed shearwater (*Puffinus tenuirostris)*, Wooller et al., 1990; Seychelles warbler (*Acrocephalus sechellen*), Komdeur, 1996). Here, we did not explore the influence of breeding experience on reproductive outcomes, but future work could categorize pairs based on breeding experience (e.g., experienced-experienced, inexperienced-inexperienced, and experienced-inexperienced), following an approach similar to Lv et al. (2016), to disentangle the effects of breeding experience and age.

The findings of our study have several important implications for the ʻAlalā conservation breeding program. First, increasing compatibility across a higher proportion of the pairs in the flock is paramount. It is possible that long-term pairings, given the monogamous mating system of the species, could increase reproductive success if the potential mates selected are indeed keen to breed. The problem of having too many moderately compatible or incompatible pairs in the program is a challenge that we hope to help resolve through ongoing mate choice studies (Greggor et al., unpublished data). Moreover, since our findings indicate that pair duration does not lead to higher productivity, birds can be more aggressively re-paired with other potential mates. While we aim to have all pairs established ahead of each breeding season, going forward, we may take a last-minute pivot approach to pairing, by finding more

suitable mates for members of newly selected pairs that are not exhibiting promising signs of breeding early in the breeding season (such as nest building or at least some potential pair bonding behaviors such as perch sharing, allopreening, and allofeeding). Moreover, we recommend that age be integrated into the ʻAlalā pair selection process, to the fullest extent possible, based on the results of this study. Although releasing pairs has been considered as a future option, there are currently no immediate plans to release established ʻAlalā pairs to the wild. Of course, reproduction in the wild comprises a vastly different system than breeding in human care; thus, we suggest that, in addition to routine monitoring, researchers study any future released pairs to understand if there are pair duration effects on reproductive success in the wild.

Our findings with ʻAlalā broadly highlight the importance of testing the effectiveness of mate selection practices in other conservation breeding programs to ensure that practitioners have the information they need to make evidence-based decisions for their unique breeding programs. It would appear that leaving unsuccessful ʻAlalā pairs together long-term takes the form of a Concorde Fallacy (i.e., sunk cost fallacy) (sensu Curio, 1987): clearly there is no empirical rationale to avoid "wasting" previous investment in establishing a pair, as it has little predictive value of future success. We suspect that many practitioners in avian conservation breeding programs with species that have similar life history characteristics as ʻAlalā are reluctant to separate mated pairs, fearing the loss of investment in developing a pair bond. We suspect equally that these decisions are too frequently made without sufficient evidence. As *ex situ* conservation assumes a more prominent role in the Anthropocene extinction crisis (Dirzo et al., 2014), it is incumbent upon us to develop efficient and effective breeding programs. These programs are costly conservation tools (Conde et al., 2011), and judicious decision-making is required before commencing an *ex situ* conservation program (McGowan et al., 2017). Once established, these programs need to produce offspring to fulfill their roles as assurance populations and as sources for translocation, or risk losing support and funding. Researchers can help bridge the science-practitioner gap (Beier et al., 2017; Greggor et al., 2021) by conducting thorough analyses of the data available in many of these breeding programs, determining what is working and what is not; this is the best path toward a more evidence-based *ex situ* conservation strategy and practice that can contribute optimally to broader conservation goals.

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 data used in this study was collected as part of the ongoing ʻAlalā conservation breeding program. ʻAlalā conservation breeding is presently conducted under U.S. Fish and Wildlife Service permit TE060179-6, State of Hawai'i Department of Land and Natural Resources permit WL21-08, and San Diego Zoo Wildlife Alliance IACUC 22-011. The animal study was approved by the San Diego Zoo Wildlife Alliance Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LB: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing, Investigation. AF: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing, Investigation. BM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing, Supervision. RS: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing, Writing – original draft.

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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 author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2024.1303239/full#supplementary-material

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The importance of quantifying fitness-determining traits throughout life to assess the application of reproductive technologies for amphibian species recovery

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The application of reproductive technologies to amphibian conservation breeding programs is gaining momentum and the field is poised to contribute significantly toward amphibian species recovery. We briefly discuss the opportunities for reproductive technologies to enhance conservation breeding outcomes, including their potential to enhance the genetic management, and in turn, the fitness of threatened species. Despite this potential, an important consideration that is not yet well understood is the degree to which specific reproductive technologies might influence (either enhance, or in some instances potentially decrease) individual fitness and lead to shifts in population viability. The development of a standardised approach to monitoring offspring throughout life-stages to detect changes to morphology, behaviour, physiology, survivorship, and developmental trajectories is essential. The primary focus of this review is to provide a 'best-practise' framework for quantifying key fitness determining traits expected to contribute to the fitness of individuals and long-term viability of populations, which will ultimately allow us to progress the field of amphibian reproductive technologies and assess the impact of protocol refinement.

KEYWORDS

amphibian, captive breeding, conservation, threatened species, reproductive technologies, biobanking, IVF, fitness traits

1 Introduction

Under the current global scenario, where species continue to decline at an unprecedented rate, integrated, multidisciplinary approaches to support species recovery are needed more than ever (Silla and Kouba, 2022). It is through an inclusive, united effort towards conservation, involving the cooperative application of a suite of strategies within

the conservationist's toolbox, that the greatest promise of success for the recovery threatened species is held. The ultimate aim of threatened species recovery is to establish robust, self-sustaining populations with enough genetic diversity and adaptive potential to remain viable over time. Reproductive Technologies (RTs, also known as assisted reproductive technologies, ARTs) are a collection of techniques within the conservationist's toolbox that have immense potential to improve reproductive outcomes, enhance genetic management, and safeguard valuable genetic resources for threatened species recovery (Silla and Byrne, 2019; Holt and Comizzoli, 2021; Bolton et al., 2022; Silla and Kouba, 2022). These technologies also afford valuable opportunities for interconnecting populations of threatened species and establishing an *ex situ- in situ* continuum (Bolton et al., 2022; Kouba and Julien, 2022). Despite the potential for reproductive technologies to assist threatened species recovery, in practice these technologies have been underutilized in amphibian conservation breeding programs (CBPs), primarily due to a need for species-specific optimisation and increased knowledge of the reproductive biology of many species required to successfully implement RTs. Over the past two-to-three decades, however, a surge in research focused on the development of amphibian reproductive technologies has progressed the field substantially and led to an increase in the application of reproductive technologies for threatened species recovery. This review begins by briefly detailing the opportunities and considerations of integrating reproductive technologies into conservation breeding programs. Next, we provide comprehensive detail on which fitness-determining traits to measure throughout amphibian life-stages in order to quantify offspring fitness and the viability of captive populations. Ultimately, the close monitoring of fitness, will allow us to progress the field of reproductive technologies and assess the impact of technological advances and protocol refinement.

2 Application of reproductive technologies to amphibian conservation breeding programs: opportunities and considerations

The application of reproductive technologies to amphibian threatened species has traditionally lagged behind that of human, agricultural and aquaculture species, however in recent years a surge of research in this field is closing the gap. Reproductive technologies encompass a complement of techniques, including those aimed at: i) monitoring the reproductive status and stress physiology of captive individuals, ii) hormone therapies to induce spawning in pairs/groups of amphibians, or induce sperm-release, or egg-release in isolated males and females, respectively, iii) cold storage for the short-term storage and transport of gametes, iv) cryopreservation and biobanking of sperm, tissue or cell lines, and v) assisted fertilisation (known as AF, or IVF). The reasons to adopt amphibian reproductive technologies and welfare considerations for their application have been recently reviewed (Silla et al., 2021), and together with numerous comprehensive technical reviews provides a guide for the accession of these technologies into conservation breeding programs (see; Kouba et al., 2013; Narayan, 2013; Vu and Trudeau, 2016; Browne et al., 2019; Silla and Byrne, 2019; Della Togna et al., 2020; Silla et al., 2021; Byrne and Silla, 2022; Clulow et al., 2022b; Graham and Kouba, 2022; Silla and Langhorne, 2022; Strand et al., 2022; Trudeau et al., 2022; Anastas et al., 2023; Upton et al., 2023). The potential for reproductive technologies to complement traditional methods of captive breeding and assist the conservation of threatened amphibians is increasingly being recognised and a steady increase in the application of RTs to conservation breeding programs is in motion.

While the application of reproductive technologies offers valuable opportunities to enhance propagation and the generation of higher numbers of individuals for assurance and reintroduction, the true value of RTs for wildlife conservation lies in their potential to enhance the genetic management of threatened species. Strategic and effectual genetic management is critical for the conservation of threatened species to prevent the loss of genetic diversity (through genetic drift and directional selection) and fitness (inbreeding depression) that typically compounds over generations and can heighten the risk of further decline (Frankham, 2008; Frankham et al., 2010). Reproductive technologies can be employed to assist genetic management by i) facilitating the generation of offspring from genetically valuable parental genotypes (through hormone therapies to induce spawning in male-female pairs, or through assisted fertilisation), ii) conducting assisted fertilisation to split clutches and ejaculates and increase the combination of parental genotypes to increase genetic diversity from a single mating event, iii) using assisted fertilisation to test for genetic compatibility and combining ability both within and between *ex situ* and *in situ* populations, iv) facilitating genetic exchange within and between *ex situ* and *in situ* populations (through gamete collection, transport and assisted fertilisation), and arguably the most powerful tool, v) employing biobanking and assisted fertilisation techniques to generate offspring from cryopreserved sperm to extend the reproductive lifespan of individuals and reinvigorate genetic diversity through the introgression of expired genotypes decades into the future (Silla and Byrne, 2019; Byrne and Silla, 2022; Anastas et al., 2023).

The enormous potential of reproductive technologies to enhance the genetic management, and in turn, the fitness of threatened species is evident. However, an important consideration that is not yet well understood is the degree to which specific reproductive technologies might influence (either enhance, or potentially decrease) individual fitness and lead to shifts in population viability. While improving and managing genetic diversity via the employment of reproductive technologies is predicted to lead to an overall increase in the fitness of populations, a standardised approach to monitoring offspring throughout life-stages to detect changes to morphology, behaviour, physiology, survivorship, and developmental trajectories are essential. An important consideration that has recently been highlighted is the potential for specific technical processes, including sperm cryopreservation, to impact fitness traits (Holt, 2023). Two primary mechanisms by which sperm

cryopreservation could impact the fitness of offspring generated from AF with frozen-thawed sperm have been suggested. First, the cryopreservation process induces artificial selection on the ejaculate, as a proportion of sperm will not survive the freeze-thaw process. This selection pressure could positively or negatively impact the fitness of resultant offspring (Nusbaumer et al., 2019). Second, the process of sperm cryopreservation and/or the nature of incubation media have the capacity to influence the transcription of genetic and epigenetic information to the developing embryo (Nusbaumer et al., 2019; Holt, 2023). There is evidence in some species of fish and amphibians that cryopreservation leads to smaller offspring size or delayed growth (Nusbaumer et al., 2019; Poo and Hinkson, 2020; Poo et al., 2022), while larger size of cryo-derived offspring has been reported in other species (Bokor et al., 2015; Lampert et al., 2022).

At present, amphibian studies investigating and reporting on the fitness of offspring generated via reproductive technologies are limited. Important considerations for designing these experiments include selecting a model amphibian lacking parental care (to control for differential investment), and possessing a large clutch size and high sperm concentration. Importantly, these characteristics will allow the use of a split-clutch/split-ejaculate breeding design to control for maternal- and paternal- effects, and parental incompatibilities. Adopting this approach will permit comparison between protocol-induced effects, while controlling for parental effects. Following from the importance of a rigorous breeding design, is the value of a standardise framework for quantifying the fitness-determining traits of offspring throughout life-stages. Multi-institutional adoption of the proposed framework provided below, coupled with transparent research reporting, is expected to progress the field of amphibian reproductive technologies and allow practitioners to assess the impact of further protocol refinement.

3 How and where to rear offspring

Amphibians typically display high levels of phenotypic plasticity, whereby the environment experienced during development interacts with an individual's genotype to alter phenotypic expression. Across a diversity of species profound changes in the timing of development and morphological features have been reported in response to heterogeneity in various environmental variables, including temperature, salinity, food availability, water level, larval density, disease, and predation risk (Newman, 1998; Parris and Cornelius, 2004; Tejedo et al., 2010; Hopkins and Brodie, 2015; Sinai et al., 2022). Consequently, it is critical that quantification of all fitness-determining traits takes place in facilities where both the rearing and test environments can be tightly controlled. We recommend that research takes place in biosecure disease-free facilities where researchers can control and monitor key environmental variables, such as temperature, humidity, water parameters, photoperiod and UV levels. As far as possible conditions should be standardised and ecologically appropriate, with decisions over what conditions to implement based on available knowledge of a study species' natural history and life history (Kelleher et al., 2018). Beyond husbandry decisions regarding essential characteristics of the environment (i.e. ensuring appropriate temperature, lighting, humidity, water, and substrate), decisions over diet and nutrition are particularly important. In addition to emerging evidence that feeding regimes (amount of food and feeding frequency) can induce amphibian developmental plasticity (Courtney Jones et al., 2015), there is a growing understanding that an individual's health, viability and performance can be significantly impacted by diet composition. In captivity, amphibians can be successfully reared to sexual maturity by feeding them a basic diet comprised of vegetable matter and fish food during the larval stage, and small cultured insects (e.g. crickets and mealworms) during the post-metamorphic life stages. However, diets lacking in key vitamins, nutrients, and micronutrients can lead to disease, developmental abnormalities and even mortality. For example, suboptimal development and performance has been linked to inadequate intake of dietary antioxidants such as Vitamin A (Ferrie et al., 2014; Rodríguez and Pessier, 2014) and carotenoids (Ogilvy et al., 2012; Silla et al., 2016), high cholesterol intake has been linked to ocular disease (Boss and Plummer, 2022), and diets deficient in calcium, phosphorous and vitamin D_3 (coupled with inappropriate provision of UVB light and low temperatures) are known to cause metabolic bone disease and neurological and musculo-skeletal abnormalities (Ferrie et al., 2014). Although species will differ in their needs, and rearing protocols will need to be developed and optimised on a species- by- species basis, in recent years detailed guidelines for the husbandry and welfare of amphibians in captivity have emerged in the literature, providing an excellent basis for developing protocols (Ferrie et al., 2014; Tapley et al., 2015; Karlsdóttir et al., 2021; Linhoff et al., 2021; Van Zanten and Simpson, 2021).

Ideally individuals should be housed separately throughout life-stages to allow the tracking of individual fitness traits (Silla et al., 2016; Byrne and Silla, 2017; Keogh et al., 2018). While there may be welfare concerns over rearing individuals in isolation, there is currently very little research to indicate that such concerns are warranted. To the contrary, there is evidence that group housing may be an acute stressor for both tadpoles (Forsburg et al., 2019) and adult frogs (Cikanek et al., 2014). Provision of life-support systems to isolated individuals, such as oxygenation of water and environmental enrichment, can be achieved through the use of automated husbandry systems (e.g. irrigation systems and rain chambers to provide clean, oxygenated water), provision of plants and tunnels/tubes (for exploration and shelter), and use of transparent enclosures to allow visual social interactions among conspecifics. If it is not feasible to rear animals individually, housing offspring in sibling cohorts and randomly selecting a subset of individuals from each cohort for the quantification of fitness-determining traits is acceptable, but large sample sizes will be needed to ensure that sufficient variation is captured. It is also recommended that detailed studbooks are maintained to record mortality, reproduction and longevity (Brereton, 2024). Tracking individuals housed within groups may be possible, but the reliability and welfare impact of different methods needs to be considered.

Microchipping, including passive integrated transponder (PIT) tags, are an option for larger amphibians (>40mm snout–vent length), but are not suitable for smaller species or tadpoles (Waudby et al., 2022). Toe-clipping is another option for juveniles and adult amphibians of some species (Waudby et al., 2022). Alternatively, tadpoles, juveniles and adults may be individually marked using visible implant alphanumeric (VIA) tags, or visible implant elastomer (VIE) markers injected subcutaneously (Fouilloux et al., 2020; Waudby et al., 2022). Each of these methods is considered invasive and animal welfare issues and potential impact on fitness requires careful consideration (Waudby et al., 2022). A less invasive approach is to draw on a species' natural external markers for photo-based identification. For example, inter-individual variation in tail venation was recently shown to be effective at identifying green and golden bellfrog tadpoles (Gould et al., 2023) and ventral patterns have been successful in identifying individual brown toadlets (Byrne and Silla, 2023). Such biometric identification can be facilitated by the use of pattern recognition software (Ribeiro and Rebelo, 2011), with recent innovations in artificial intelligence and deep learning providing the opportunity to train software to identify individuals with extreme precision (Takaya et al., 2023).

4 What fitness-determining traits should be measured?

Multiple traits contribute to total fitness, that is the probability that an individual (population, or species) successfully reproduces and contributes genes to future generations. Therefore, practitioners should aim to measure a suite of traits that represent different components of fitness (e.g. survival or fecundity), as well as traits that are indirectly related to fitness (e.g. body size or growth rate) (see Figure 1). Below we discuss traits that are likely to be most critical to fitness in amphibians, and, in turn, those that are critically important in regulating population size, growth rate and persistence. We also detail the types of methods and assays required to ensure accurate and biologically relevant measurements are taken.

4.1 Fertilisation success

Among sexually reproducing animals, including amphibians, fertilisation is controlled by the interaction of gamete recognition proteins located on the surface of sperm and eggs (Tian et al., 1997). Incompatibilities between these proteins (reflecting genetic incompatibilities) can cause significant drops in fertilisation success, with direct implications for reproductive success and fitness in both sexes. Fertilisation success can be estimated within hours of AF by scoring the proportion of eggs that have rotated. Eggs are characterised by two hemispheres; the animal pole (typically darker in colour) and the vegetal pole (typically lighter in colour). When a sperm penetrates an egg, the axis between the poles will rotate to align with the gravitational field, resulting in the animal pole facing upwards. The only downside of this approach is that rotating eggs can sometimes cause unfertilised neighbouring eggs to rotate, inflating estimates of fertilisation success. To avoid this problem, fertilisation success should be confirmed using optical microscopy. To ensure a standardised approach, eggs should always be staged using generalised staging systems (Gosner, 1960). In anurans fertilisation is normally scored between the 2-cell stage (Gonser stage 3) and the neural-plate stage (Gosner stage 13). Not all studies score fertilisation success, but this is a mistake because it can lead to inaccurate estimates of early embryo mortality (Sagvik et al., 2005). Where possible, eggs should be removed from terrariums/aquariums as soon as possible following oviposition and fertilisation success scored (a dissection microscope can aid in looking for cell division). Amphibian eggs are generally quite robust due to their protective jelly coat, so using sterile plastic spoons and tweezers to carefully manipulate the eggs to view and score fertilisation is possible.

4.2 Embryo survival and embryo abnormalities

Among amphibians it is well established that mortality risk is highest during embryonic and larvae life stages (Matthews et al.,

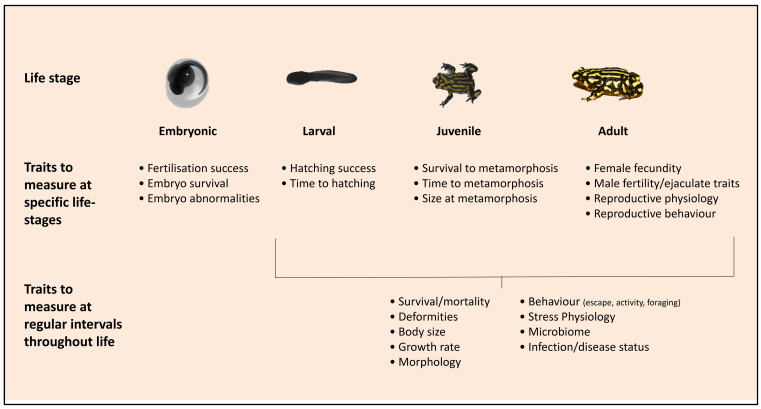

FIGURE 1
Important traits to measure that represent different components of fitness in amphibians.

2013), and that changes to this risk can influence population persistence (Biek et al., 2002). Genetic incompatibilities that reduce offspring viability are expected to manifest, and compound, during early development. Therefore, it is important to continuously monitor developing embryos and score exactly when mortality occurs (using generalised staging systems to record the stage of death). Importantly, in certain species, development can be suspended prior to hatching (embryonic diapause). Therefore, embryos should only be discarded once they show clear signs of death. Embryos should also undergo routine pathology to monitor fungal and viral pathogens (Wright and Whitaker, 2001). For example, aquatic oomycetes (known as "water molds") can cause the disease saprolegniosis, which is known to infect wild and captive amphibian embryos. Infertile and dead eggs are readily infected with saprolegniosis postmortem (Costa and Lopes, 2022). Developing embryos are also known to be susceptible to infection if they have been exposed to trauma (physical or chemical) to the protective egg jelly capsule, or through contact infection between adjacent eggs (Costa and Lopes, 2022). Reducing the risk of infection during embryonic development is another reason that rearing offspring individually from the point of fertilisation is encouraged. Where clutches are kept together, they should be monitored regularly for embryo mortality and infection and embryos removed that exhibit symptoms. Surviving embryos should also be monitored for abnormalities that provide an indication of genetic incompatibility. Common embryonic disorders and malformations include oedema, severely bent trunk axis, axial flexures and shrunken, enlarged, or missing eyes and tails.

4.3 Hatching success and time to hatching

Scoring the proportion of eggs hatching is a reliable way to estimate offspring viability. Hatching failure is common in amphibians, and generally indicates that embryos have died just prior to hatching, or have fatigued and died during the strenuous process of exiting the egg capsule. Hatching in amphibians is normally triggered by a specific environmental cue (e.g. vibration or hypoxia), and is also typically asynchronous. As such, before eggs are scored as failed, they should be checked for signs of life (e.g. reflex movement or a heartbeat) and decomposition. Hatching success per family should be scored by calculating the number of eggs successfully hatched as a proportion of the number of eggs fertilised. The time between stimulation of hatching and successful hatching can also be used to score time to hatching. Time to hatching can have important fitness consequences as individuals that hatch faster are expected to access resources required for larval development more rapidly. The only caveat here is that there may be a tradeoff between time to hatching and body size at hatching (Stearns, 1992). If individuals that hatch quickly are smaller, this could have flow on affects that negatively impact other developmental milestones that influence fitness (time to metamorphosis and size at metamorphosis). The potential for such life history tradeoffs, and flow on effects, should be considered on a species-by-species basis as this may inform decisions regarding optimal rearing conditions.

4.4 Larval survival, deformity, growth rate, body size and shape

Mortality in amphibians can be extreme during the larvae phase as many individuals fail to thrive. Therefore, this is a critical time to measure survivorship. Of note, mortality at this life-stage may be due to numerous reasons that are often undetermined. Histopathology and/or necropsy to attempt to determine cause of death of tadpoles is encouraged and full procedural details are provided elsewhere (Wright and Whitaker, 2001). Larval survival can be measured at specific time points (e.g. daily or weekly intervals) or specific developmental points (e.g. emergence of the first hind limb or forearm). Larval viability can also be assessed by recording the appearance of deformities, which are expected to compromise survival and fitness (see Pakkasmaa et al., 2003). Abnormalities normally present as lateral, concave or convex curvature of the spine (scoliosis, lordosis, kyphosis), abnormal accumulation of fluid in tissue (oedema), malformed heads or tails, missing tails, or missing, shrunken or misplaced eyes. Photos can be taken of tadpoles (next to a scale) at regular intervals, and images analysed using image-analysis software. Sophisticated landmark based geometric morphometrics offer a powerful way to quantify variation in morphology in amphibians (see Vidal-García et al., 2018), but even very basic measures of head and tail length and width and total length will allow estimates of variation in body size and shape (see Pakkasmaa et al., 2003; Rudin-Bitterli et al., 2018, 2020). These measures can be informative because tadpole size often correlates with size at metamorphosis (Pakkasmaa et al., 2003). Moreover, tadpole shape is known to influence ability to escape predators (Relyea, 2001), which is one of the most important sources of mortality during the larval stage in the wild (Wells, 2010). Taking photos at defined time points also provides the opportunity to measure growth rate, which is often inter-related with time to metamorphosis and body size at metamorphosis. From a practical perspective, it is best to photograph tadpoles *in situ* to avoid handling stress. This can often be achieved by taking the photograph from above, after dropping the water to avoid size distortions as tadpoles move through the water column, and placing grid paper under the container to provide a scale for measurement (Keogh et al., 2018; Mcinerney et al., 2019). If dropping the water level prior to capturing photographs, it is important that tadpoles remain fully submerged, and movement is unrestricted (a minimum depth of 2–5cm depending on tadpole size) to avoid adverse impacts on amphibian welfare. Water levels in each aquarium should be returned to normal immediately after photographs have been taken (Keogh et al., 2018). Where tank design doesn't allow photographs from above, removal of tadpoles may be necessary. This can be achieved using a fine mesh fish net and placing tadpoles in an individual water dish under a camera set up, noting that tadpoles are very delicate in the first week or two following hatching.

4.5 Survival to metamorphosis and metamorph deformities

Metamorphosis is a physiologically demanding transformation and is typically accompanied by a spike in mortality, so survival to

metamorphosis is a good indicator of viability. Survival can be measured at various points, but the most common are; 1) the point of complete forelimb emergence (Gosner stage 42), 2) the point when individuals leave the water (though this can be hard to monitor), and 3) the point of complete tail resorption (Gosner stage 46) (Rudin-Bitterli et al., 2018; Székely et al., 2020; Weber et al., 2024). Of note, metamophs can easily drown, so care should be taken to drop water levels and provide a suitable substrate to allow metamorphs to exit the water. Failing to do so can mask genetic causes of mortality. As metamorphs grow, they should be checked for possible congenital abnormalities, such as lateral deviation of the spine (scoliosis), polymelia (presence of extra limbs), ectromelia (missing limbs), ectroddactly (missing digits), ectopic appendages, missing or misplaced eyes, microcephaly (shrunken head), bicephaly (presence of two heads), and shortened lower jaw (brachygnathia). Definitions of the diversity of deformities observed in amphibians, and practical guide to their identification and causes, have been detailed elsewhere (Meteyer, 2000; Lunde and Johnson, 2012).

4.6 Time to metamorphosis and body size at metamorphosis

It is a central tenant of life history theory that individuals entering adulthood faster, and at a larger body size, will have higher lifetime fitness, and be selectively favoured (Stearns, 1992). In line with this notion, there is evidence that TTM and SAM are effective predictors of post-metamophic survival, fecundity, and performance in various amphibians (Pakkasmaa et al., 2003; Earl and Whiteman, 2015). Broadly speaking, however, meta-analysis has indicated that SAM is a much better fitness predictor than TTM, and that the value of these measures as measures of fitness appears to be highly species and system specific (Earl and Whiteman, 2015). For instance, there is evidence that SAM predicts fitness more accurately in species in which there is a shorter amount of time between metamorphosis and reproductive maturity (Earl and Whiteman, 2015). Nevertheless, we recommend that TTM and SAM are routinely recorded because they can be measured quickly and accurately and have the potential to be highly informative (especially if the life history and ecology of the target species is well known). TTM can be measured at various points (see above) and body size can be estimated by weighing individuals immediately after tail resorption to estimate body mass, and using callipers or photos to measure snout-vent length (SVL). Mass-length relationships can also be used to calculate a body condition index (Maccracken and Stebbings, 2012), which can be an important fitness determinant in amphibians. Saying this, if time and funds permit, more direct measures of body composition (e.g. lipids, proteins, fatty acids, amino acids, micronutrients) may provide a better estimate of the resources available to support metabolic activity, or other fitness related characters, such as muscles mass (Wilder et al., 2016).

4.7 Adult survival

As animals age there will be progressive mortality, and we should expect that rates of mortality will be higher for individuals of poor genetic quality (Dziminski et al., 2008). As such, there is value in keeping careful records of when individuals die. Histopathology and/or necropsy to determine cause of death is encouraged and full procedural details are provided elsewhere (Wright and Whitaker, 2001). We also recommend the postmortem extraction and biobanking of gonadal tissue, including gonadal cell lines, testes macerates, or oocytes (Kaurova et al., 2021; Clulow et al., 2022a; Strand et al., 2022).

4.8 Adult body size

Across amphibians, there is a growing body of evidence that larger individuals have a greater chance of survival, which has been linked to impacts on risk of desiccation, infection, predation, starvation, and competition for resources (Wells, 2010; Cabrera-Guzmán et al., 2013). There is also evidence for strong positive relationships between body size and reproductive success, largely due to relationships between body size and fecundity and fertility, as well as between body size and attractiveness in mate choice, and success in male-male competition (see Amézquita and Lüddecke, 1999; Kupfer, 2007; Wells, 2010). As such, recording body size (ideally as weight and SVL) for all experimental animals at regular time intervals during adult life (in addition to larval and juvenile life-stages) is recommended. Keeping a photographic record of body size throughout life-stages is also encouraged as this provides the opportunity for morphometric analysis of body traits linked to fitness (see Vidal-García et al., 2018). It is also important to note that sexual size dimorphism (SSD) is strong in amphibians, with female-biased SSD reported in 91% of anurans, 79% of salamanders, and 81% of caecilians (Pincheira-Donoso et al., 2021). As such, it is recommended that body size and growth rate be quantified for each sex independently (see Linhoff et al., 2022 for a review of methods for identifying sex in amphibians).

4.9 Female fecundity and male fertility

Fecundity is known to be a significant contributor to lifetime reproductive success in amphibians. For some species fecundity will correlate tightly with body size (allowing body size to be used as proxy), but this won't always be the case. As such, if animals are reared to sexual maturity, we advise keeping long term records of clutch number and clutch size produced for each female. For males, the non-invasive collection of spermic urine, milt, or spermatophores is encouraged (Silla and Langhorne, 2022), though if animals die unexpectedly or need to be euthanized for welfare reasons, testes should be extracted post-mortem to generate testes macerates (Silla et al., 2017; Kaurova et al., 2021). Following sperm collection, we advise measuring a range of characteristics known to influence fertilisation capacity and male reproductive success in amphibians, including sperm concentration, viability (proportion live/dead, DNA integrity), motility parameters (velocity, percentage motility, forward progression, longevity), and morphology (head length, tail length, morphological

deformities). Detailed methods for the measurement of these sperm traits are provided in Silla and Langhorne (2022).

4.10 Behavioural and physiological performance

Animal behaviour, the measurable response of an individual to external or internal stimuli, plays a crucial role in determining fitness (an individual's ability to survive, reproduce and pass genes to the next generation). Assays of escape performance, exercise/locomotory performance, foraging performance, general-movement behaviour and sexual display, can all provide estimates of individual viability. The behavioural ecology literature is replete with examples of these types of assays, and they range from being very simple and easy to run, to highly complex (Kelleher et al., 2018). Some relatively simple tests include observing or video recording test subjects and measuring sensorimotor performance, such as quantifying foraging efficiency (Mcinerney et al., 2016), locomotory performance (Székely et al., 2020), activity levels in familiar home environments (Videlier et al., 2014; Urszán et al., 2015), and exploration and boldness behaviour in novel environments (Brodin et al., 2013; Carlson and Langkilde, 2013; Kelleher et al., 2019). For many species, anti-predatory escape responses (quantified as escape burst speed and distance travelled) can also be measured following a simulated predator attack. This is commonly achieved by imposing a light physical stimulus to larvae or adults to induce a flight response (Touchon and Wojdak, 2014), or by imposing either; 1) a chemical threat stimulus, whereby a test subject is presented with chemical stimuli associated with a predator (Sih et al., 2003; Wilson and Krause, 2012; Carlson and Langkilde, 2014) or 2) a physical threat stimulus, whereby a test subject is threatened by a live or model predator (Koprivnikar et al., 2012; Urszán et al., 2015; Kelleher et al., 2017; Mcinerney et al., 2017). More complex assays include the use of robotics (Klein et al., 2012), sophisticated 3D animations (Bian et al., 2018), behavioural tracking software and remote videography (Hughey et al., 2018) to standardise stimuli and capture subtle or long term inter-individual behavioural differences in foraging behaviour, movement behaviour, anti-predatory behaviour, and mating behaviour (reviewed in Kelleher et al., 2018). In regard to physiological performance, the capacity to withstand fatigue during sustained activity (endurance) provides a good proxy for dispersal ability and is often easily measured by running larvae or adults in linear or circular race tracks and recording the time and distance moved (Herrel et al., 2014; Araspin et al., 2023). When testing adults, researchers will also often measure fatigue using a 'righting ability test', whereby individuals are flipped on their back and the time to right is recorded (Herrel et al., 2014; Silla et al., 2016). Beyond such tests, increasingly sophisticated physiological measures of performance can also be highly informative. One widely used approach is to measure basal metabolic rate, defined as the overall energetic costs required to fulfil physiological needs (Padilla et al., 2023). Basal metabolic rate is accurately measured using a respiratory chamber and flow throw respirometry and is strongly linked to fitness due to direct impacts on growth, development, reproduction, and general functioning (Santos and Cannatella, 2011; Padilla et al., 2023). Another increasingly used physiological test involves measuring the maximal jump force of frogs (linked to performance in dispersal, foraging, predator escape and competition), quantified using a jump plate and charge amplifier (Herrel et al., 2014; Araspin et al., 2023). It is also important to recognise that there can be sex-specific differences in behavioural performance (Herrel et al., 2014), making it essential to quantify experimental variables for both sexes independently.

There is also a growing interest in non-invasive methods for measuring stress physiology (Narayan et al., 2019; Narayan, 2022). In captive contexts, sources of stress can include excessive handling, overcrowding, competitive interactions/aggression, overstimulation (e.g. from external stimuli such as vibration, light and sound), and lack of refugia (Ferrie et al., 2014). With the advent of non-invasive methods for monitoring of acute stress in captive amphibians, such as quantifying urinary, faecal, water-borne, and dermal secretions (Cikanek et al., 2014; Santymire et al., 2018; Forsburg et al., 2019; Narayan et al., 2019; Narayan, 2022), it is now possible to track the stress responses of individuals in various contexts (including in response to the behavioural assays described above). Of note, knowledge of causes of stress can also be used to optimise husbandry protocols and rearing conditions (see section 3. above).

5 Conclusion

Reproductive technologies encompass a complement of techniques aimed at understanding and enhancing reproductive outcomes and genetic management, and the application of these technologies to amphibian conservation breeding programs is gaining momentum. In order to assess the degree to which RTs influence individual fitness, and to allow practitioners to assess the impact of protocol refinement, a standardised approach to monitoring the fitness-determining traits of offspring throughout life-stages is critical. Acquiring this information will help conservation practitioners run cost-benefit analyses to inform decisions surrounding the integration of reproductive technologies into the recovery plans for declining species.

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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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Visualizing the risk landscape to adaptively increase post-release survival of translocated Galliformes

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Translocation of captive-bred animals is a widely used conservation strategy to support the recovery of imperiled wild populations. Identifying which factors enhance or limit survival after release can be important in adapting translocation strategies, particularly for species with low survival rates after release from captivity. Many translocation programs track post-translocation survival, but few complete spatial-statistical assessments of mortality risk associated with release environments. Typically, few animals are released from captive breeding programs, limiting the sample size available for analyses. We aimed to create a workflow that used limited datasets to evaluate the influence of spatial conditions and other factors on mortality risk. Greater sage-grouse (*Centrocercus urophasianus*) are endangered in Canada and of conservation concern throughout their range in the United States. After the species declined precipitously in Canada, a captive breeding program was initiated with subsequent releases in Alberta and Saskatchewan. Despite success in captive breeding, mortality rates of released sage-grouse were high. We used GPS- and VHF-based locations of released sage-grouse to determine how spatial features influence mortality risk of sage-grouse after release from captivity. We implemented a multistep approach to quantify and map risk relative to the environmental features associated with mortality. We also assessed whether the movement behaviors of sage-grouse correspond with environmental risk factors by using a combination of survival models and integrated step-selection functions. Mortality of sage-grouse in Alberta was hastened in areas close to anthropogenic disturbance. Although birds in Alberta avoided areas of higher mortality risk, those in Saskatchewan did not, perhaps due to environmental and selection constraints. This multistep approach allowed us to utilize small sample sizes to assess key risk factors in the landscape. This process supports the adaptive modification of translocation plans and can similarly support other data-limited scientists and managers in assessing environmental mortality risk and defining conservation actions for endangered species.

KEYWORDS

accelerated failure time model, Anderson-Gill model, conservation translocation, Cox proportional hazards model, captive breeding, greater sage-grouse, integrated step-selection function, risk surface

1 Introduction

Wild environments are dynamic and complex. As natural environments change, conservation practitioners increasingly need to make high-stakes decisions with available environmental and population information, and analyses that make use of limited data (Armstrong and Seddon, 2008). Conservation translocation programs often operate within this decision and information space. Translocation planners make decisions on where to release animals and how to modify the release environment at the onset of the program with the information available, which is often uncertain. Plans can be modified as information on outcomes are collected, but the adaptive feedback loop can be slowed or stalled by the challenges of using small datasets.

Conservation translocations are often used to relocate animals and bolster populations on the brink of extirpation (Snyder et al., 1996; Berger-Tal et al., 2020) and have helped conserve many threatened species (Seddon et al., 2014). Conservation translocations (hereafter, translocations) benefit species at imminent risk of decline and extinction by moving organisms from one location to another, and include reintroductions (e.g., into the historical range from which a species has been extirpated) and (or) as reinforcements (e.g., to augment extant populations, IUCN/SSC, 2013; Seddon et al., 2014). Translocations generally require a high degree of conservation effort (e.g., time and resources, Fischer and Lindenmayer, 2000), involve a high degree of uncertainty, and result in varying degrees of success (Griffith et al., 1989; Fischer and Lindenmayer, 2000). Translocation program success is often difficult to measure because this assessment requires long-term data and standardized criteria success, both of which are often lacking (Morris et al., 2021; Marino et al., 2024). Success of translocations is frequently gauged based on environmental (e.g., habitat quality, improvement, or range; predation; competition), species-specific (e.g., reproductive potential, migratory tendency, diet, survival, genetic diversity), methodological (e.g., origin and number of released individuals, program duration, release method, age at release), and other factors (e.g., public perception and support, stakeholder relationships, program management, and long-term commitment to the project) (Rummel et al., 2016; Marino et al., 2024). Evidence of translocation success has been seen in a variety of species including the Arabian oryx (*Oryx leucoryx*; Al Jahdhami et al., 2011), California condor (*Gymnogyps californianus*; Parish and Hunt, 2016), Lord Howe Island woodhen (*Hypotaenidia sylvetris*; Frith, 2013), Mallorcan midwife toad (*Alytes muletensis*; Bloxam and Tonge, 1995; IUCN, 2018), natterjack toad (*Bufo calamita*; Denton et al., 1997), duskytail darter (*Etheostoma percnurum*; Shute et al., 2005), American burying beetle (*Nicrophorus americanus*; Amaral et al., 1997), and swift fox (*Vulpes velox*; Moehrenschlager and Moehrenschlager, 2001; Cullingham and Moehrenschlager, 2013). However, translocation success rates have not generally increased, despite significant advances in translocation research (Bubac et al., 2019). Captive breeding and release exemplifies a high-risk, high-cost strategy and this approach is used when few other conservation options remain (Ferrer et al., 2018; Berger-Tal et al., 2020).

Captive breeding and release programs often aim to create and sustain a captive assurance population by adding animals that can survive and reproduce to wild populations (McCarthy et al., 2012; Seddon et al., 2012). These programs have successfully restored several imperiled populations to pre-endangered numbers (e.g., peregrine falcon, *Falco peregrinus*, Tordoff and Redig, 2001; Mauritius kestrel, *Falco punctatus Temmink*, Nicoll et al., 2004). Yet, many programs, have difficulty producing animals that survive long enough to reproduce in wild populations. Among other reasons, survival can be low because translocated animals are introduced to an unfamiliar landscape and lack knowledge of locally-relevant mortality risks (Bell, 2016). They may select poor-quality or sink habitats (Le Gouar et al., 2012), move through areas of high predation risk, and make poor dietary choices (Berger-Tal et al., 2020).

The release environment can greatly influence survival and the success of translocations to wild populations (Osborne and Seddon, 2012). Release environments are heterogeneous and present mortality risks such as spatially varying predator abundance, sources of collision and disturbance, and movement, food, and cover constraints (Le Gouar et al., 2012). An understanding of the mortality risks associated with the release environment can be integral to the success of translocation programs (IUCN/SSC, 2013), by leading managers to optimize potential release locations or other environmental conditions (Moehrenschlager and Lloyd, 2016). However, quantifying which environmental factors present mortality risk can be challenging.

In the absence of more complete information, characterizations of habitat are often expected to represent places that support the survival of released animals (Osborne and Seddon, 2012). Managers may also use informal or formal expert-driven approaches to assess environmental risk in the release area by evaluating the ability of the environment to support the species' life history and resource needs (e.g., habitat maps or other proxy information), and local knowledge of predators and threats (Nichols and Armstrong, 2012). By weighing and mapping these factors, managers can select release areas and sites to minimize mortality risk. These assumptions or hypotheses of mortality risk factors and locations facilitate timely action. However, translocation and release plans could be adapted over time with survival data collected from the first few years of release programs, and spatial-statistical analyses that test habitat-risk assumptions and expert hypotheses.

Quantitative risk analyses for translocated animals are difficult to do with small sample sizes of released animals and a myriad of potential sources of environmental risk, dissuading translocation practitioners from pursuing spatial-statistical analyses (Armstrong and Seddon, 2008). Generally, a small number of animals are released and most die soon after (Teixeira et al., 2007), leaving few records to evaluate factors associated with longer-term survival. Prior to death, animals experience a range of risks as they traverse the landscape (Nichols and Armstrong, 2012). Evaluating the risk factors associated with the journey, rather than simply the location of death or the site of transmitter recovery, presents another challenge.

In this paper, we provide an example of a multistep workflow that leverages limited animal location data to characterize the risk landscape. We developed this workflow using a Galliform listed as endangered by the Canadian Species at Risk Act (SARA), the Greater sage-grouse (*Centrocercus urophasianus*). We first constrained the list of potential environmental factors by conducting univariate analyses on all hypothesized environmental risk factors, then built multivariate models with all key or significant variables to understand the factors most influencing time to mortality. We included the species' habitat map as a potential covariate to test the expectation that habitat areas with higher selection values confer lower mortality risk. We used a spatial survival model that assesses risk using the location history of each animal and mapped the resulting multivariate model to visualize the risk landscape. Subsequently, we used a step-selection function to assess the congruence of movement behavior with mapped mortality risk by evaluating movement speeds, turn angles, and movement-selection decisions by animals relative to the risk map. This workflow yielded an understanding of which environmental features are associated with shorter and longer survival times, the locations of key risk areas, and how released animals behaved in response to those features. We discuss how these analytical results could lead to new spatial strategies to improve post-release survival in terms of release sites and changes to environmental conditions. We also discuss whether environmental changes that reduce mortality risks are likely to be perceived by an animal and result in different movement behavior.

2 Materials and methods

2.1 Study species and area

Galliformes are challenging to keep alive and breed in captivity (Lance et al., 1970; Flieg, 1971; Johnsguard, 1983; Hancock, 1993), and their post-release survival and integration into wild populations has proved to be even more difficult (Parker et al., 2012; Carrlson et al., 2014; Mathews et al., 2016). Translocations of captive-bred Galliformes to wild environments generally result in few individuals surviving to reproduce the subsequent year (Sokos et al., 2008; Wiechman et al., 2011; Collar, 2020). Most die within a few weeks post release, primarily from predation (Parish and Sotherton, 2007; Merta et al., 2016), partly as a result of poor predator avoidance behaviors of inexperienced birds (Rantanen et al., 2010). High post-release mortality may also be due to suboptimal habitat selection and lower fitness (Rantanen et al., 2010), physiological stress during both pre- and post-release periods (Dickens et al., 2009), and exposure to weather, malnutrition and (or) starvation (Thompson et al., 2015). When captive-bred Galliformes do survive to reproduce, they can produce fewer offspring relative to their wild counterparts (e.g., 0.05 hatched chicks per released female vs. 2.09 hatched chicks per wild female, Putaala and Hissa, 1998) due to hen and egg predation (Putaala and Hissa, 1998; Parish and Sotherton, 2007) and suboptimal habitat selection and higher nest abandonment (Sage et al., 2003).

Galliformes, especially Greater sage-grouse; hereafter, sage-grouse), are known to reside in suboptimal locations where they may not perceive risk and modify their movements to optimize their fitness (Aldridge and Boyce, 2007; Heinrichs et al., 2018; Pratt and Beck, 2021). Hence, knowledge of how sage-grouse navigate through the risk landscape could reveal mismatches in perceived and actual risk (Pratt and Beck, 2021). This information may be useful in avoiding the assumption that translocated sage-grouse perceive risk as mapped or confirming they are responding to risk in a novel wild environment (Parker et al., 2012). This information would also inform and direct recovery planning and decision-making regarding habitat management that would optimize resources and maximize conservation gain (i.e., focus improvements where most valuable).

Like other Galliformes, sage-grouse were historically abundant throughout North America, but declined precipitously during the 20th century due to habitat conversion, degradation, fragmentation, and reduction of sagebrush (*Artemisia* spp.) (Knick et al., 2003; Connelly et al., 2004; Aldridge et al., 2008). In Canada, only two small isolated populations remain in Alberta and Saskatchewan, and sage-grouse have declined by up to 92% in the last ~30 years (Environment Canada, 2014). As such, sage-grouse are considered endangered in Canada under the Canadian SARA (Environment Canada, 2014), prompting an urgent response including a captive breeding and release program to augment wild populations. Adaptive improvements to both the program and release environment are ongoing to support the recovery of sage-grouse in Canada. Habitat restoration, reclamation, infrastructure removal, beneficial grazing, and a multitude of other practices have been identified in recovery plans and implemented on the ground (AESRD, 2013; Parks Canada Agency, 2021).

Sage-grouse are sagebrush-obligate species and use sagebrush throughout much of their life cycle for food and cover. Although the species once flourished in Canada, human-induced changes to the environment (e.g., vegetation, predator guild, disturbance) have contributed to sage-grouse population declines. Sage-grouse currently exist in a mosaic of public and private lands in Canada, and their survival across the species' range is affected by a range of environmental factors and stressors including anthropogenic features (e.g., energy infrastructure, roads, buildings; Aldridge and Boyce, 2007), agricultural development and activities (Beck et al., 2006), predators (Tack, 2009), and insufficient habitat (e.g., inadequate sagebrush, shrub and grass cover; Connelly et al., 2004; Gregg and Crawford, 2010; rugged terrain, Beers and Frey, 2022). Large portions of the species' habitat and current range are protected, and critical habitat has been identified and mapped using a resource selection function (Environment Canada, 2014). In Canada, sage-grouse critical habitat includes areas with large

patches of moderate and spatially-dispersed shrub cover (e.g., silver sagebrush), minimal bare ground, moderately moist habitats with some amount of lush green vegetative cover, adequate availability of forage forbs, as well as areas with lower intensity of anthropogenic disturbance (e.g., human settlements, roads, fences, annual cropland, artificial structures for avian predators (Environment Canada, 2014).

We assessed mortality risk using animal location and known fate data from captive-bred sage-grouse that were released at two release sites (near Manyberries, Alberta; and west block of Grasslands National Park near Val Marie, Saskatchewan; Figure 1). Both study sites are within the mixed-grass prairie ecoregion (Alberta Parks, 2015; Parks Canada, 2022), where silver sagebrush (*A. cana*) is the dominant shrub species (see Supplementary Materials for further details).

2.2 Threats

In Alberta and Saskatchewan, sage-grouse habitat includes several potential stressors or risks including avian predator perches (e.g., trees, powerlines, fence posts), habitat loss (e.g., lack of shrub cover), and environmental disturbance (e.g., energy infrastructure, increased vehicle traffic) (Environment Canada, 2014). Cattle grazing occurs across much of the sage-grouse range on private lands, provincially designated grazing leases, and federally designated grazing areas. The Alberta and Saskatchewan study areas differ in the composition and spatial distribution of risk factors. For instance, much of the sage-grouse habitat in Alberta has been affected by oil and gas extraction activities, whereas anthropogenic infrastructure is relatively limited in Grasslands National Park in Saskatchewan (Government of Saskatchewan, 2023). In Grasslands National Park, habitat is concentrated in the valley bottom, and limited in the West block of the park where sage-grouse were released (Thorpe and Stephens, 2017), with escarpments separating lowland and flat upland habitats. By contrast, sage-grouse habitat occurs across a larger area with varied topography in Alberta.

2.3 Captive rearing and release

Juvenile sage-grouse were released in Canada from 2018 to 2022 (n = 228), in Alberta (n = 119) and in Saskatchewan (n = 109). Sage-grouse were weighed, sexed, and tagged with transmitters before release and tracked until death or signal loss. Many sage-grouse mortalities occurred soon after release and proximate to the release site. These individuals had little opportunity to move away from the release site and select landscape features in the risk landscape. As our aim was to spatially understand the environmental covariates contributing to longer survival, we restricted analyses to individuals that survived at least 10 days after release. This also minimized the noise associated with the post-release 'settling down' period (Musil et al., 1993).

We categorized mortality as early (e.g., survived ≤ 10 days post release) and late (e.g., survived > 10 days post release), based on median survival days. We used the median survival as a threshold for including individuals (and all associated movement records) in the analysis, and exclusion of individuals that died too early to experience much of the post-release landscape. We used the median rather than average survival, because most sage-grouse died shortly after release, resulting in a right-skewed distribution. When carcasses were found, a cause of death was determined with either high or low confidence by biologists at the Wilder Institute at the Calgary Zoo (Supplementary Table 1). We analyzed bird location data separately for each province of release and built models with both spring and fall release seasons combined, and only fall releases (i.e., in September and October).

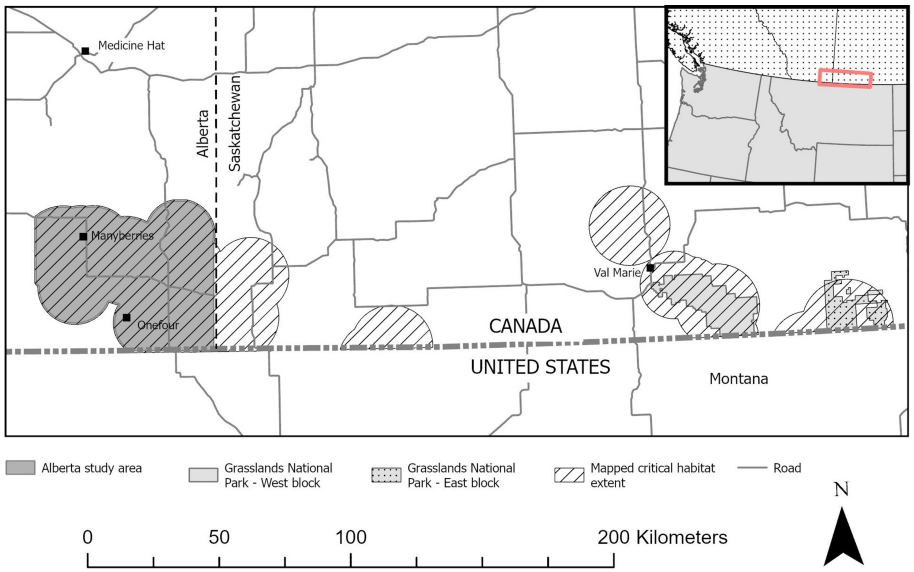

FIGURE 1
Two study sites where captive-bred Greater Sage-grouse (*Centrocercus urophasianus*) were released from 2018 to 2022 in Alberta and Saskatchewan, Canada, and where mortality risk, habitat selection, and movement were evaluated post release.

2.4 Environmental covariates

To assess survival risk relative to environmental features, we developed a range of spatial data layers to use as covariates in our models. We selected similar types of covariates for each study site (Supplementary Tables 2–4) and developed layers to describe each family of covariate (e.g., anthropogenic disturbance, cover of shrubs and sagebrush, critical habitat, release site, roads, terrain, vegetation, vertical features, water). We included a map of critical habitat (Environment Canada, 2014), derived from a sage-grouse nest occurrence model for Alberta (Aldridge and Boyce, 2007), that was extrapolated to Saskatchewan. The resource selection function included covariates describing mean shrub cover within 1 km^2, proportion of patchily distributed shrub cover within 1 km^2, greenness and wetness indices, and relationships to roads and other built-features.

For each spatial variable, we derived proportion, proximity, or cover layers, summarized at various spatial scales to evaluate the scale of influence of each variables. For example, we summarized proportional data using two moving window sizes that are biologically relevant to sage-grouse. In Alberta, we tested survival responses to proportional covariates using windows sizes of 0.55 km, the median daily distance traveled by birds released in Alberta between 2018 and 2022, and a 1-km survival-infrastructure responses from previous research (e.g., Aldridge and Boyce, 2007). In Saskatchewan, we similarly evaluated the median daily distance traveled by birds released in Saskatchewan (2.3 km), and tested responses using a 0.56 km window used in other sage-grouse studies (e.g., Aldridge and Boyce, 2007; Fedy et al., 2014; Kirol et al., 2015; Parsons et al., 2022). We evaluated the predictive ability of each scale in univariate mortality risk analyses and retained the scale of the covariate that had the lowest Akaike Information Criteria corrected for small sample size (AICc, Burnham and Anderson, 2002).

Our primary focus was assessing spatial features influencing sage-grouse mortality risk; however, species traits (e.g., sex) and release features (e.g., timing, age at release) can also influence mortality (Swenson, 1986; Beck et al., 2006; Maness and Anderson, 2013). We included the following aspatial covariates in statistical analyses: acclimation period (e.g., > or < 14 days), age at release, body mass, raising method (e.g., hen- and hand-raised), release period (e.g., early release before October 17, and late release after October 17), release type (e.g., hard, modified soft, and soft), and sex (see Supplementary Materials for hypotheses).

2.5 Statistical analyses

We implemented a four-step approach to assess sage-grouse movements and mortality risks associated with environmental covariates (Figure 2). In step one, we conducted a univariate screening between covariates and mortality risk, to reduce the number of covariates entering multivariate models to accommodate sample size constraints. Prior to fitting models, we assessed correlation among covariates (Pearson's $r \geq |0.70|$). We standardized covariates by subtracting the covariate mean from the individual value and dividing it by the standard deviation. In step two, we built multivariate models of remaining covariates to assess time to mortality. In step three, we selected the top performing models and applied the multivariate equation to spatially referenced covariates to create a risk surface map. In step four, we fit a step-selection function using the sage-grouse GPS location data, risk surfaces (from step three), and existing critical habitat maps.

2.5.1 Univariate mortality risk model

In step one, we fit univariate Cox proportional hazards models (hereafter, Cox models; Figure 2) to assess which spatial covariates had the strongest independent influences on mortality risk, using the *coxph* function within the *survival* package in R (Therneau, 2023). This variable reduction step was necessary because we were constrained by a limited sample size of location and mortality data and needed to reduce the number of covariates entering multivariate models. We right-censored data from sage-grouse with unknown fates at the last known date alive and used midpoint-interval censoring to estimate the date of mortality for sage-grouse that did not have a known mortality date (Kalbfleisch and Prentice, 1980). We tested the proportional hazards assumption in Cox models by plotting the scaled Schoenfeld residuals for each variable against time (e.g., Johnson et al., 2004) using the *cox.zph* function within the *survival* package in R (Therneau, 2023). For models that violated the proportional hazards assumption, we stratified models by time (e.g., year, Therneau and Grambsch, 2000). We also assessed deviance residuals to identify potential outliers using the *ggcoxdiagnostics* function, along with evaluating Martingale residual plots to assess nonlinearity in covariates (Fox and Weisberg, 2018). For continuous covariates that violated the assumptions of linear effects on the logarithm of the hazard, we fit Cox models with smoothing splines, and assessed if there were significant differences (e.g., p-value > 0.05) among linear and spline Cox models using a likelihood ratio test (Mahboubi et al., 2011). If there were no significant differences between linear and spline Cox models, we proceeded with the linear Cox model. If spline-based Cox models outcompeted the linear Cox model but did not pass the proportional hazards test, we used the recommended alternative modeling approach of Accelerated Failure Time models (hereafter, AFT models; Saikia and Barman, 2017), using the *flexsurspline* function in the *survival* package in R (Jackson, 2016). Because AFT models require an assigned distribution of survival times, we compared several distributions (e.g., Weibull, lognormal, log-logistic) and selected the best fit distribution based on the lowest AICc (Burnham and Anderson, 2002; Saikia and Barman, 2017). We fit Cox and AFT univariate models to determine the covariates that strongly influenced mortality risk (e.g., 95% confidence bounds did not overlap zero), and compared models to their respective null model without covariates. We retained covariates within our nine main covariate categories (e.g., anthropogenic disturbance, cover of shrubs and sagebrush, critical habitat, release site, roads, terrain, vegetation, vertical features, water) that were strong predictors and (or) that outcompeted other covariates within the same covariate

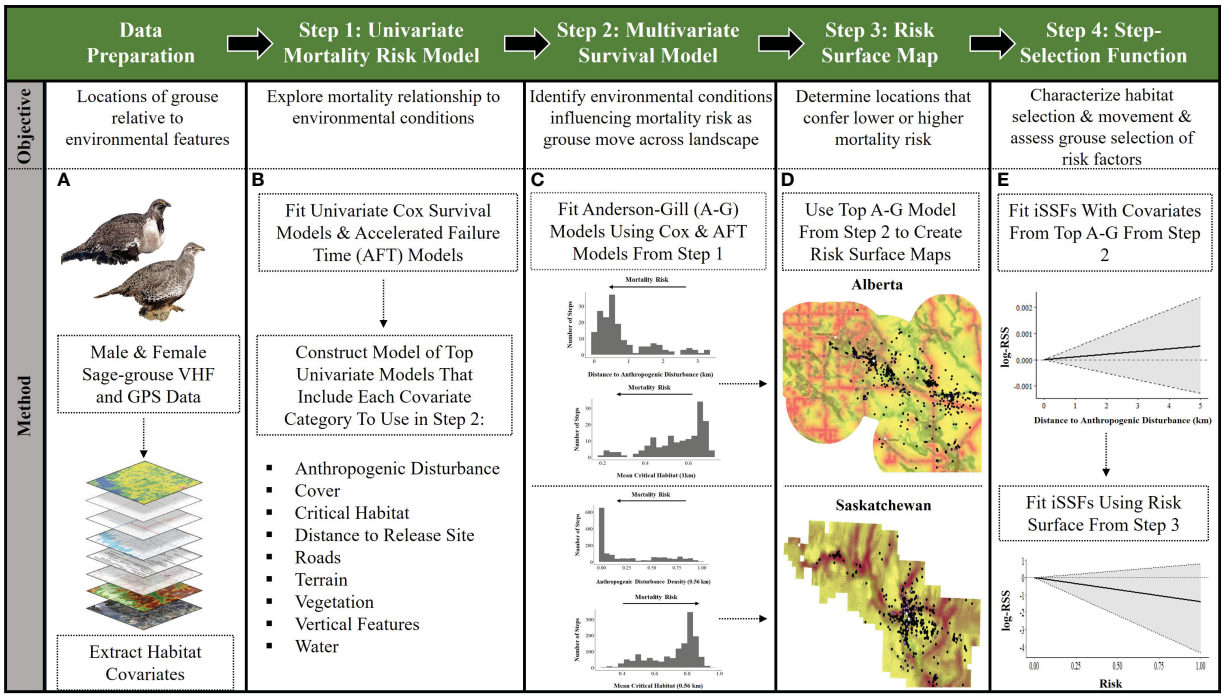

FIGURE 2
Steps taken to assess mortality risk, movement, and selection of mortality risk factors, for captive-bred and released Greater sage-grouse (*Centrocercus* urophasianus) in Alberta and Saskatchewan, Canada (2018–2022). Panel **(A)**, VHF and GPS data from male and female sage-grouse are prepared by extracting habitat covariates at each location, including mortality locations. Panel **(B)**, the data are then analyzed using univariate Cox Proportional Hazards and Accelerated Failure Time (AFT) models to explore relationships among environmental features and mortality locations (Step 1). Then, top univariate models (e.g., covariate 95% confidence bounds did not overlap zero) of the main covariate categories (e.g., anthropogenic disturbance, cover, critical habitat, distance to release site, release site, roads, terrain, vegetation, vertical features, water) were used to construct multivariate models due to sample size constraints. Panel **(C)**, multivariate models were then fit with Andersen-Gill (A-G) models to identify which environmental conditions contribute to mortality risk as sage-grouse move across the landscape (Step 2). Plots depicted in Step 2 show the relationship between number of steps (locations) in relation to covariates in top A-G models for each Canadian province. Panel **(D)**, top A-G models (from Step 2) are used to create risk surface maps from unstandardized β-coefficients applied to a logistic equation in ArcGIS Pro (Step 3). Panel **(E)**, integrated step-selection functions (iSSFs) were fit with covariates from top A-G models (from Step 2) to characterize habitat selection and movement (Step 4). The first plot in Step 4 indicates a step is less likely to end in areas relative to distance to anthropogenic disturbance. Finally, iSSFs were fit using the risk surface from Step 3 to assess if grouse are selecting habitat based on perceived risk (Step 4). The second plot in Step 4 demonstrates sage-grouse are more likely to avoid risky habitat compared to what is available. Photos of Greater sage-grouse © Cornell Lab of Ornithology.

category (e.g., anthropogenic disturbance density within 1 km, proportion of development within 1 km). We combined the best-performing covariate (e.g., based on covariate 95% confidence bounds not overlapping zero or if not significant, based on lowest AICc of univariate models) in each of the nine covariate categories to create multivariate survival models.

2.5.2 Multivariate survival model

In step two, we used Andersen-Gill survival models (hereafter, survival models) to assess how covariates contribute to sage-grouse mortality risk as grouse move throughout the landscape (Figure 2). Andersen-Gill models explain how survival rates change with a given covariate (Johnson et al., 2004). These survival models are an extension of Cox models, that model entry date (i.e., starting time step), exit date (i.e., end time step), and an event for each encounter of an individual, where sample size is based on the number of events (i.e., encounter locations, Andersen and Gill, 1982). Andersen-Gill models can accommodate right-censored data (i.e., individuals with unknown fates, Johnson et al., 2004). Each event was coded as 1 for mortality or 0 for unknown or alive (i.e., right-censored data), using the *coxph* function within the *survival* package in R (Therneau, 2023). We treated each time interval between sequential locations as a distinct risk interval that accounts for continuous time, but with differing intervals. We also stratified models by bird band number. Spatial covariates were extracted at the end of each time interval (e.g., end time step, Johnson et al., 2004; Therneau, 2023). We used Cox and AFT models that included at least one variable from each covariate family, then modeled all combinations of these covariates in multivariate survival models using the *dredge* function in the *MuMIn* package in R (Barton, 2018). We identified the best multivariate survival model as the model with the lowest AICc (Burnham and Anderson, 2002).

We evaluated goodness of fit of top survival models via likelihood ratio tests χ^2 (Johnson et al., 2004). We also assessed top survival model predictability with Concordance statistics for which values > 0.5 indicate predictive power greater than by chance alone, and values closer to 1.0 indicate good model fit and predictive power (Therneau, 2023). Resulting positive β-coefficients from survival models were associated with increased hazard (i.e., hastened mortality), and negative β-coefficients indicated

decreased hazard (i.e., delayed mortality). We reported risk ratios, or exponentiated β-coefficients from top survival models, which indicate the influence of a covariate on sage-grouse mortality (Johnson et al., 2004), where values >1 suggest increased risk. In step three, we created risk surface maps from our top survival models using the unstandardized β-coefficients (applied to a logistic equation in ArcGIS Pro) to calculate the predicted relative probability of sage-grouse mortality in each 30 x 30 m pixel, for both Canadian provinces. In each risk surface, we restricted all continuous spatial covariates to the maximum values where sage-grouse were observed, to ensure we were not predicting beyond the scope of survival models that were used to estimate β-coefficients.

2.5.3 Step-selection function

In step four, we assessed the degree to which sage-grouse demonstrate movement and selection patterns that are consistent with mortality risk maps (Figure 2). We characterized movement relative to the risk landscape using an integrated step-selection function (hereafter, iSSF; Avgar et al., 2016). Following existing iSSF methods that assess habitat selection and mortality risk, we used the *amt* package in R (Signer et al., 2019), to use movement location records to jointly assess habitat selection and movement direction and step length (Avgar et al., 2016). ISSFs utilize conditional logistic regression (Thurfjell et al., 2014; Panzacchi et al., 2016) to compare the habitat characteristics between two successive locations (i.e., a 'step'; Turchin, 1998); locations are defined as used (by birds; coded as 1) and available (could be used; coded as 0). Each step taken by the bird is scored by the iSSF, with the higher score indicating a higher probability of being selected (Fortin et al., 2005). We generated 100 random available steps per observed step, with random step lengths drawn from a recommended gamma distribution (Avgar et al., 2016) and random turn angles drawn from a von Mises distribution (Duchesne et al., 2015). To meet the assumption of relatively constant time intervals (Signer et al., 2019) we resampled the data to form regular bursts using the function *track_resample* function in the *amt* package (Signer et al., 2019), which allowed us to partition the steps into groups of observations with a sample rate of one day. The choice of the one-day sampling rate was based on limitations from the GPS and VHF data collection (i.e., daily location data). Based on early assessment of Used-Habitat Calibration (UHC) plots (Fieberg et al., 2018), we only retained individual datasets that included >30 locations. We also stratified the conditional logistic regression model by the bird band number to ensure used and available locations for each bird were properly aligned (Fieberg et al., 2021).

We first used covariates in our top survival models in our iSSF analyses to characterize movement, and then fit iSSFs to the risk surface we created in step three (Figure 2) to evaluate the degree of correspondence in movement behavior and mapped mortality risk. We evaluated selection of key environmental covariates, and included step length, the logarithm of step length, and the cosine of the turning angle as covariates in the model to account for underlying movement processes (Avgar et al., 2016). We used inverse-variance weighted regression to summarize inference from individual models at the population level by calculating mean parameter estimates and 95% confidence intervals around them. Each parameter value expresses relative selection strength on the log scale (log-RSS, Avgar et al., 2017) for a one-unit increase of the corresponding covariate, where positive values express selection and negative values express avoidance. The log-RSS indicates how many times more likely a step is to end in risky habitat (along the x-axis) versus the mean risk value. We also used empirical bootstrapping to estimate 95% confidence intervals around mean parameter values. Under a presence-background study design, available points are not necessarily unused, rather, their status is unknown; for this reason, standard metrics, such as area under the curve (AUC, Brown and Davis, 2006) are also not appropriate for this situation (Fieberg et al., 2018). Thus, we used calibration plots designed for use-availability data (Boyce et al., 2002; Fieberg et al., 2018) to further evaluate model performance. Given we extracted separate spatial information pertaining to each province, we did not pool the data across Alberta and Saskatchewan, and thus provide the following results by province.

3 Results

3.1 Sage-grouse mortalities

Female sage-grouse generally had a higher number of confirmed mortalities than males, likely due to females being easier to track (e.g., had larger, more powerful transmitters than males), but typically lived longer than males (Supplementary Tables 5, 6). Time to mortality for females varied between provinces. With few exceptions, sage-grouse died within 20 days of release in Alberta and within 26 days in Saskatchewan (Figures 3A, C). Only 30% of males and ~58% of females survived past 10 days after release in Alberta; and a greater proportion of the released population survived past 10 days in Saskatchewan (50% of males, ~78% of females; Supplementary Tables 5, 6, respectively). Only two hens survived more than one year in our study sites (e.g., Alberta hens = 873, and 1,185 days – dropped transmitter). These outliers were omitted from analyses, but we found both hens primarily used less risky habitat (mean risk = 0.10, SD = 0.07, maximum risk value = 0.36, n = 152 locations; mean risk = 0.13, SD = 0.06, maximum risk value = 0.34, n = 207 locations; respectively) compared to other sage-grouse that survived at least 10 days (mean risk = 0.23; SD = 0.15; maximum risk value = 0.75; n = 1,220 locations). Most individuals had known fates (Supplementary Tables 5, 6). Unknown survival status resulted from transmitter failure or dropped devices. Location data were used in analyses until the time the signal was lost (see Multivariate Survival Model methods above).

Predation accounted for most confirmed deaths in both study sites (Figures 3, 4; Supplementary Table 2). Carcasses were examined by biologists and the predators associated with mortalities were identified as mammal (e.g., canids such as coyotes, *Canis latrans*) and avian species (e.g., raptors such as great horned owls, *Bubo virginianus*) in both study sites (Figure 4). Confirmed mortalities were also caused by weather (exposure) in both provinces. There was a notable snowstorm in Alberta in November 2020, which resulted in the accumulation of

~45 cm of snow. The extreme, extended temperatures (e.g., -20°C on ~90% of nights during a 2-week period in December 2021 and January 2022) and predation contributed to sage-grouse deaths in Alberta. Mortalities were also caused by collisions with vehicles and fences in Alberta only (Figure 4; Supplementary Table 2).

3.2 Univariate mortality risk model

We fit 83 univariate Cox proportional hazards and Accelerated Failure Time models for Alberta (Supplementary Table 9), which resulted in nine covariates that moved into multivariate survival models (see Table 1 for subset of candidate models). Of those nine covariates, distance to gravel roads and streams strongly influenced mortality in Alberta (Table 1 and Supplementary Table 9). For Saskatchewan, we fit 61 univariate Cox proportional hazards and Accelerated Failure Time models (Supplementary Table 13), which resulted in 10 covariates that moved into multivariate survival models (see Table 1 for candidate models only). Of those 10 covariates, anthropogenic disturbance density within 0.56 km, proportion of meadow and other shrubs within a median daily distance travelled (2.35 km), elevation, and distance to roads and tall features strongly influenced mortality in Saskatchewan (Table 1 and Supplementary Table 13). In univariate tests, acclimation period and raising method had weak predictive ability and were not retained in multivariate survival models (Supplementary Tables 7–19; Figures 1, 2). Later release dates (e.g., after October 17), influenced mortality risk, but only in Saskatchewan (Supplementary Table 13).

3.3 Multivariate survival model

We initially started with a list of spatial and aspatial covariates in our multivariate survival models, but spatial factors outcompeted non-spatial covariates (Supplementary Tables 7–19; Figures 1, 2). In the Alberta multivariate survival model, we included 46 sage-grouse (10 males and 36 females, n = 878 total locations) (Table 1). The top multivariate survival model included distance to anthropogenic disturbance, which strongly influenced sage-grouse mortality risk (Table 1). We found mortality risk strongly decreased by 62% per 1-km increase in distance to anthropogenic disturbance (risk ratio: 1.00 – exp(β) = 0.38, SE = 0.44, 95% CI = 0.17, 0.88, Table 2). The proportion of critical habitat (within a median daily distance travelled; 0.55 km) had a weak effect (Tables 1, 2). Mortality risk was weakly influenced by lower mean critical habitat values within 1 km and was reduced by 18% per 1-unit increase in mean critical habitat (risk ratio: 1.00 – exp(β) = 0.82, SE = 0.21, 95% CI = 0.57, 1.18, Table 2, Figure 5 and Supplementary Figure 3). The top survival model fit reasonably well (Concordance statistic = 0.64; Likelihood Ratio Test: χ^2 = 7.05, df = 2, p-value = 0.03).

In the Saskatchewan multivariate survival model, we included 52 sage-grouse (12 males and 40 females, n = 1,508 total locations; Table 1). Our top survival model (Tables 1, 2) indicated that mortality risk was strongly influenced by both mean critical habitat and anthropogenic disturbance density within 0.56 km. Mortality risk decreased by 37% per 1-unit increase in anthropogenic disturbance density (risk ratio: 1.00 – exp(β) = 0.63, SE = 0.23, 95% CI = 0.41, 0.97, Table 2) and increased by 82% per 1-unit increase in mean critical habitat (risk ratio: exp(β) = 1.82, SE = 0.22, 95% CI = 1.28,

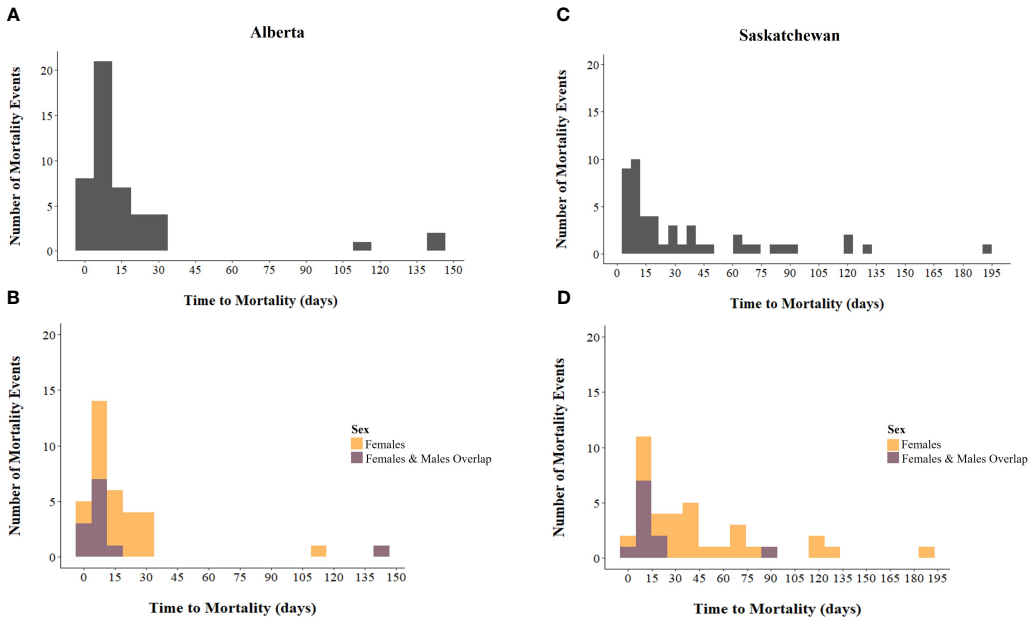

FIGURE 3
Number of mortality events of male (light purple) and female (orange) released, captive bred Greater sage-grouse (*Centrocercus urophasianus*) during spring and fall combined in: **(A, B)** Alberta (n = 47; male, n = 12, female, n = 35), and **(C, D)** Saskatchewan (n = 48; male, n = 11; female, n = 37), Canada, 2018 – 2022, by time to mortality (days). Mortality events where sexes overlap **(B, D)**, are shown in deep purple, and time to mortality (days) is grouped into 15-day bins.

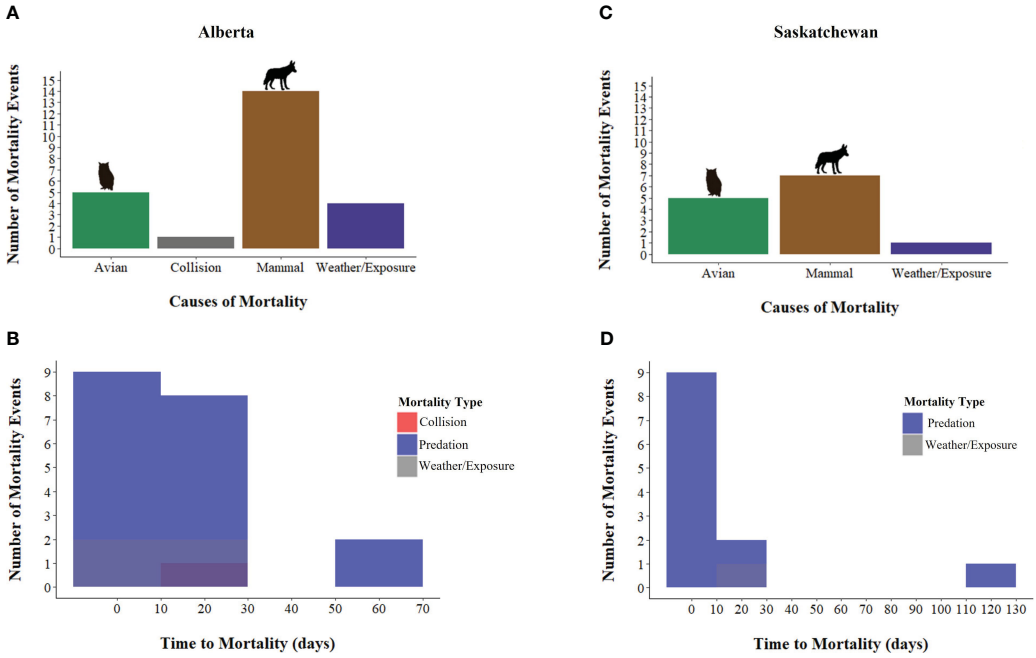

FIGURE 4
Known causes of mortality of released, captive bred Greater sage-grouse (*Centrocercus urophasianus*) in: **(A)** Alberta (n = 25), and **(C)** Saskatchewan (n = 13), Canada, 2018 – 2022, and number of mortality events through time in (grouped into 10-day bins): **(B)** Alberta, and **(D)** Saskatchewan.

2.59, Table 2). The model exhibited good model fit (Concordance statistic = 0.70; Likelihood Ratio Test: χ^2 = 9.53, df = 2, p-value = 0.01). The survival model and associated risk surface mapped higher risk of mortality in areas with lower densities of anthropogenic disturbance and higher mean critical habitat densities within 0.56 km (Figure 6 and Supplementary Figure 4).

3.4 Step-selection functions

We examined iSSF calibration plots and determined we could fit iSSFs to seven individuals (n = 900 locations) in Alberta and 20 individuals (n = 406 locations) in Saskatchewan. In both provinces, nearly all birds maintained a relatively straight path or made only slight changes in direction when step lengths represented daily steps (negative turn angle; Alberta: β = −0.49 ± 0.31; Saskatchewan: β = −0.53 ± 0.19). In the Alberta iSSF, the selection coefficient for distance to anthropogenic disturbance remained around zero, denoting that at a mean step length (3.23 km), anthropogenic disturbance was neither selected nor avoided by sage-grouse in general (Figure 5C). In Alberta, numerous birds selected areas with higher proportions of habitat (mean critical habitat within 1 km; β = 3.93 ± 2.69; Figure 5C). We also found evidence that sage-grouse made movement decisions that minimize exposure to risky areas compared to what is available to them (β = −8.34 ± 4.08, Figure 7A). By contrast, in Saskatchewan, a step was more likely to end in greater anthropogenic disturbance density (β = 1.54 ± 0.81) and in greater mean critical habitat both within 0.56 km (β = 2.41 ± 2.49), compared to average values (mean step length = 1.18 km) (Figure 6C). Additionally, the population in Saskatchewan appears to be more likely to select for risky habitat compared to what is available (β = 1.08 ± 1.14, Figure 7B).

3.5 Mortality around release sites

Release sites and surrounding areas differed in terms of their proximate mortality risks (Figure 8). The release sites (30-m pixels) were generally located in sage-grouse habitat with higher pixel scores in the critical habitat layer and were in locations associated with lower mortality risk (i.e., northwest release site in Alberta, Figure 8A; both release sites in Saskatchewan, Figure 8C). The southeast release site in Alberta was in a location with lower-scored habitat, with lower mortality risk (Figure 8B). However, the areas surrounding release sites included areas of high mortality risk, with patterns and risk levels that differed among sites. For example, the two Alberta release sites had very different mapped risks (Figures 8A, B), suggesting that post-release survival could be different between these places. Although we did not have the sample size to compare survival between these two locations, future studies may amass enough data to do so.

4 Discussion

4.1 Mortality risk

We assessed how environmental factors can influence the mortality risk of captive-bred sage-grouse that were released into the wild. Mortality risk of birds that survived longer than 10 days was

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TABLE 1 Subset of candidate Anderson-Gill survival models of late mortality risk (e.g., died > 10 days post release), number of parameters (*k*), Akaike's Information Criterion (AICc) scores, differences among AICc scores (Δ), and AICc weights (w_i) for released, captive bred Greater sage-grouse (*Centrocercus urophasianus*) in Alberta and Saskatchewan, Canada, 2018 – 2022.

Location	Model	k	AICc	ΔAICc	w_i
Alberta	Distance to Anthropogenic Disturbance + Mean Critical Habitat†	2	136.38	0.00	0.29
	Distance to Anthropogenic Disturbance + Mean Critical Habitat† + Badlands Binary + Distance to Release Site	4	137.13	0.75	0.20
	Mean Critical Habitat† + Distance to Release Site + Distance to Gravel Roads	3	138.13	1.75	0.12
	Distance to Anthropogenic Disturbance + Proportion of ≥ 20% Shrub Cover + Mean Critical Habitat†	3	138.36	1.98	0.11
	Distance to Anthropogenic Disturbance + Mean Critical Habitat† + Badlands Binary	3	139.07	2.69	0.08
	Mean Critical Habitat† + Distance to Release Site	2	139.16	2.79	0.07
	Mean Critical Habitat† + Distance to Gravel Roads	2	140.42	4.05	0.04
	Distance to Anthropogenic Disturbance + Proportion of ≥ 20% Shrub Cover + Mean Critical Habitat† + Badlands Binary + Distance to Release Site	5	140.43	4.06	0.04
	Mean Critical Habitat† + Distance to Streams	2	140.59	4.22	0.04
	Mean Critical Habitat† + Distance to Release Site + Distance to Gravel Roads + Elevation	4	141.01	4.63	0.01
	Null	1	151.92	15.54	0.00
Saskatchewan	Anthropogenic Disturbance Density§ + Mean Critical Habitat§	2	210.17	0.00	0.24
	Anthropogenic Disturbance Density§ + Distance to Waterbodies + Mean Critical Habitat§	3	210.74	0.57	0.18
	Anthropogenic Disturbance Density§ + Distance to Tall Features + Distance to Waterbodies + Mean Critical Habitat§	4	211.41	1.24	0.13
	Distance to Waterbodies+ Mean Critical Habitat§	2	212.46	2.29	0.08
	Anthropogenic Disturbance Density§ + Mean Critical Habitat§ + Distance to Release Site	3	212.54	2.37	0.08
	Anthropogenic Disturbance Density§ + Distance to Waterbodies + Mean Critical Habitat§ + Distance to Release Site	4	212.66	2.49	0.07
	Proportion of Other Shrubs‡ + Mean Critical Habitat§	2	212.72	2.55	0.07
	Proportion of Other Shrubs‡ + Distance to Waterbodies + Mean Critical Habitat§	3	212.80	2.63	0.06
	Anthropogenic Disturbance Density§ + Distance to Tall Features + Distance to Waterbodies+ Mean Critical Habitat§ + Distance to Release Site	5	213.05	2.88	0.06
	Proportion of Meadow‡ + Distance to Waterbodies+ Mean Critical Habitat§	3	213.07	2.90	0.03
	Null	1	258.36	48.19	0.00

†values extracted within a buffer radius = 1 km, representing a value shown to be predictive in other Greater sage-grouse studies.
§values extracted within a buffer radius = 0.56 km, representing a common scale among other Greater sage-grouse studies.
‡values extracted within a buffer radius 2.35 km, representing median daily distance traveled in Saskatchewan.

influenced by anthropogenic disturbance and habitat factors in both Alberta and Saskatchewan, although responses were somewhat nuanced and different between these places. In Alberta, anthropogenic disturbance was the most influential factor influencing mortality risk. Increased exposure to areas proximate to anthropogenic disturbance substantively hastened sage-grouse mortality (Table 2), as demonstrated by elevated risk ratios. As predators often use anthropogenic infrastructure for perching,

TABLE 2 Environmental covariates of Greater sage-grouse (*Centrocercus urophasianus*) mortality, after release from captivity into Alberta or Saskatchewan, Canada (2018 – 2022), estimated from top Andersen-Gill survival models using location records of birds that survived at least 10 days.

Location	Covariate	β	SE	95% CI	Risk Ratio		
					exp(β)	SE	95% CI
Alberta	**Distance to Anthropogenic Disturbance**	-0.96	0.44	(-1.79, -0.13)	0.38	0.44	(0.17, 0.88)
	Mean Critical Habitat †	-0.20	0.21	(-0.56, 0.16)	0.82	0.21	(0.57, 1.18)
Saskatchewan	**Anthropogenic Disturbance Density**§	-0.46	0.23	(-0.89, -0.04)	0.63	0.23	(0.41, 0.97)
	Mean Critical Habitat §	0.60	0.22	(0.25, 0.95)	1.82	0.22	(1.28, 2.59)

Results presented are standardized coefficients (β) and the associated standard errors (SE) and 95% confidence intervals (CI), and risk ratio [exp(β)] and the associated SE and 95% CI. Covariates in bold indicate a strong response on mortality risk (e.g., 95% confidence intervals of β do not overlap zero).
†values extracted within a buffer radius = 1 km, representing a value shown to be predictive in other Greater sage-grouse studies.
§values extracted within a buffer radius = 0.56 km, representing a common scale among other Greater Sage-grouse.

hunting, or movement (Connelly et al., 2000; Hagen et al., 2011; Baxter et al., 2013), higher mortality in these areas was expected and observed in other studies (Lammers and Collopy, 2007; Prather and Messmer, 2010; Slater and Smith, 2010; Dinkins et al., 2014; Howe et al., 2014; Dinkins et al., 2016). Anthropogenic disturbance can mask approaching predators (e.g., noise from disturbance, Hovick et al., 2014), and can also cause avoidance of areas by sage-grouse, which has been previously documented in southeastern Alberta (e.g., Aldridge and Boyce, 2007). For example, translocated sage-grouse are known to select habitats further from anthropogenic disturbance, up to 10 and 15 km from buildings and settlements, respectively, and up to 2.5 km from roads (Balderson, 2017). Wild sage-grouse in Alberta also avoided oil and gas infrastructure up to 1.9 km from energy development during winter (Carpenter et al., 2010), but did not consistently select habitats that increase survival (Aldridge and Boyce, 2007; Heinrichs et al., 2018). In Alberta, the mortality of wild sage-grouse chicks increased in areas with a higher visible well density within 1 km (Aldridge and Boyce, 2007). We observed a similar mortality response for adult birds released from captivity at the 1-km scale but did not have the sample size to include individual anthropogenic features in the multivariate model. We combined anthropogenic features (i.e., paved and gravel roads, residences, industrial developments, *and* active wells; Supplementary Table 2); hence, our results did not identify the specific built features that most impact sage-grouse survival. However, univariate models indicated distance to gravel roads were more influential than other anthropogenic features (Supplementary Table 9). Future analyses could assess and compare singular

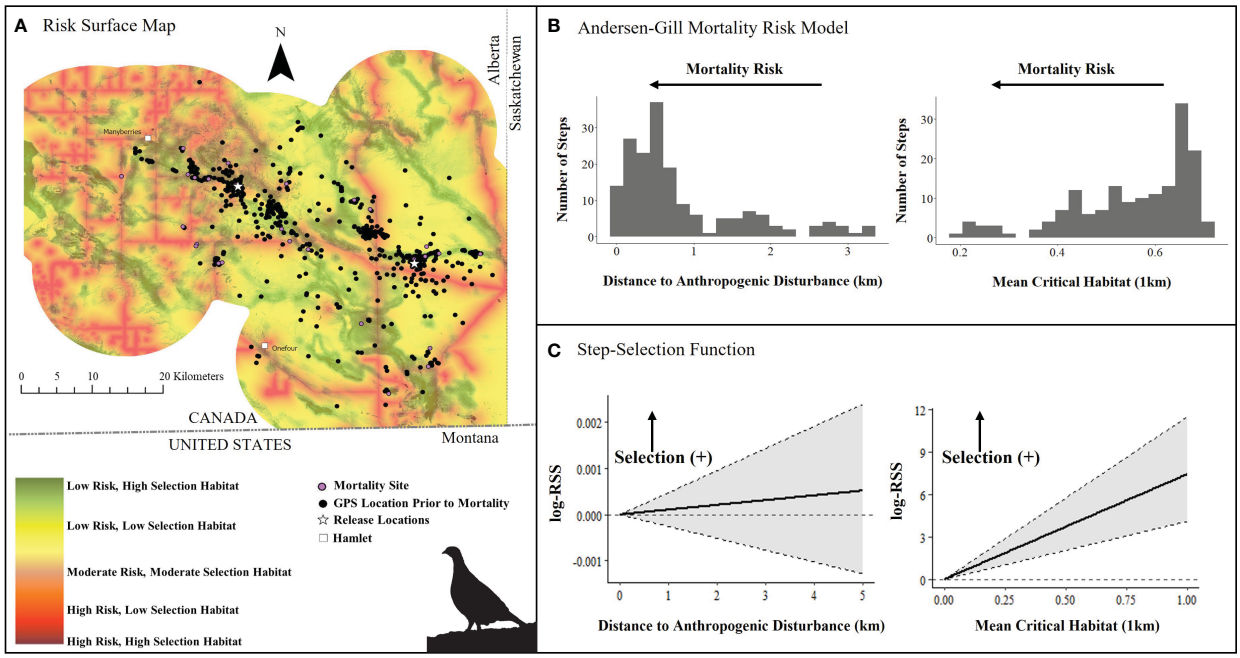

FIGURE 5
(A) Mortality risk surface map of captive bred Greater sage-grouse (*Centrocercus urophasianus*) released in southeastern Alberta during spring 2021 and 2022 and fall 2018 – 2021, layered on critical habitat probability (Environment Canada, 2014), **(B)** number of steps (locations) associated with each covariate in top Andersen-Gill model, and **(C)** quantified resource selection strength estimated by an integrated step-selection function. Silhouette of Greater sage-grouse © Cornell Lab of Ornithology.

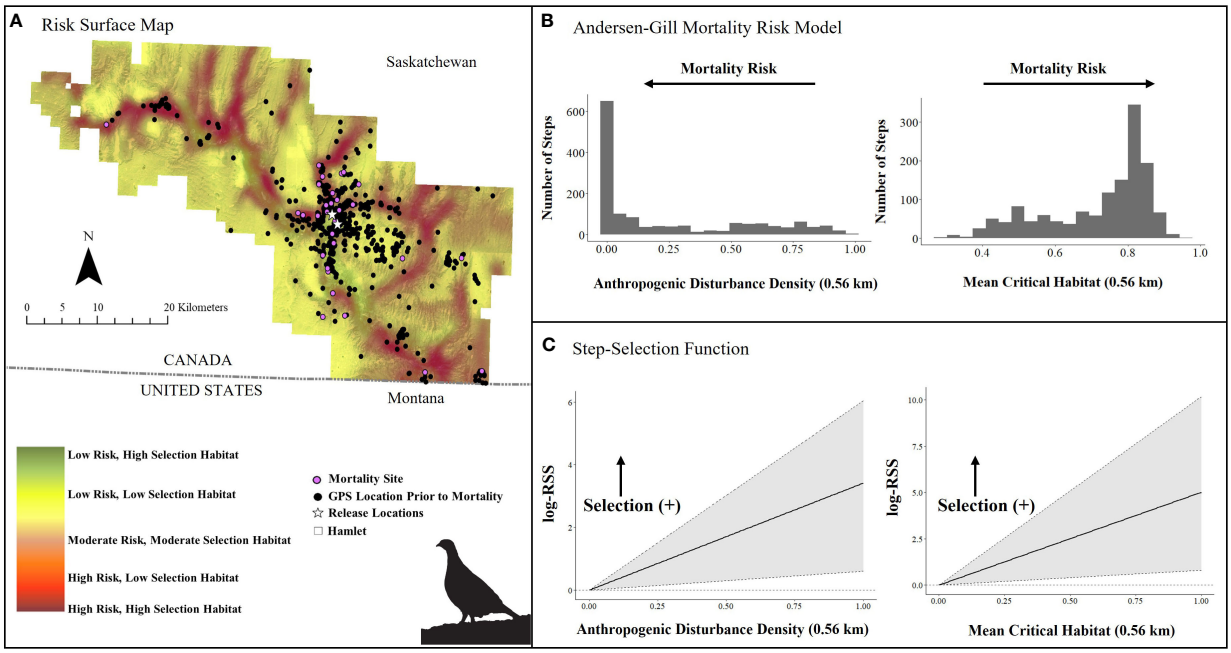

FIGURE 6
(A) Mortality risk surface map of captive bred Greater sage-grouse (*Centrocercus urophasianus*) released in southwestern Saskatchewan during spring 2021 and 2022 and fall 2018 – 2021, layered on critical habitat probability (Environment Canada, 2014), **(B)** number of steps (locations) associated with each covariate in top Andersen-Gill model, and **(C)** quantified resource selection strength estimated by an integrated step-selection function. Silhouette of Greater sage-grouse © Cornell Lab of Ornithology.

anthropogenic features (e.g., only roads) to help managers understand which features have the greatest influence on mortality.

In Saskatchewan, anthropogenic features and habitat were key variables but their relationships to mortality differed from those in Alberta. Contrary to expectation, sage-grouse that used areas with lower densities of anthropogenic disturbance (within 0.56 km) and higher-scored habitat (proportion of mean critical habitat in 0.56 km moving window) were associated with hastened times to mortality (Table 2). This result may be a consequence of the unique environmental context in Saskatchewan. Areas occupied by released, captive-bred sage-grouse tended to be located in the valley bottoms where there is a low density of development, a high proportion of high-selection habitat, and near water resources and higher densities of shrub cover. Predators may be attracted to these productive areas and predation may be high despite the lower availability of anthropogenic features. Thus, mortality may be high in low disturbance areas with high selection habitats because of predators making similar resource choices.

Although environmental risk factors were comparable among release provinces, the differences in mortality risk for sage-grouse underscores that mortality risk can be context dependent. The risk factors were different in each region of release, and these risks were heterogeneously distributed across the landscape, resulting in local and regional maps that could be used as decision criteria in subsequent plans for selecting release sites and regions. Although habitat should be expected to contribute to sage-grouse survival (as observed in Alberta), habitat and associated maps can be an insufficient indicator of mortality risk or of the quality of a release site and its local environment. Habitat maps can provide an initial guess of where to release captively-bred animals; however, these guesses may be misleading when other environmental factors that influence survival are not included and mapped in habitat analyses. We included environmental variables that represent opportunities for predation, including anthropogenic and tall features, topography, and restricted cover, but lacked spatial information that described predator abundance and distribution. When available, this information could be used to augment spatial risk analyses and help further identify the potential causes of mismatches in habitat and survival conditions (e.g., O'Neil et al., 2020).

4.2 Risk responses and movement

We found mixed evidence that released sage-grouse respond to environmental conditions in a way that minimizes mortality risk. The step-selection function results for sage-grouse released in Alberta suggest they generally moved and selected locations that were in less risky habitat compared to what is available to them (Figure 7A). They selected for higher-scored habitats (greater mean critical habitat; Figure 5C), further away from anthropogenic disturbance (although not significant; Figure 5C) and these choices resulted in a slower or decelerated time to mortality (Table 2; Figure 5). Avoidance of anthropogenic features is consistent with how wild sage-grouse select habitats within various critical life history stages (e.g., nesting, brood-rearing, Aldridge and Boyce, 2007) and seasons (e.g., breeding, summer, winter, Gelling, 2022). However, sage-grouse do not always optimize fitness with habitat selection (e.g., maladaptive selection;

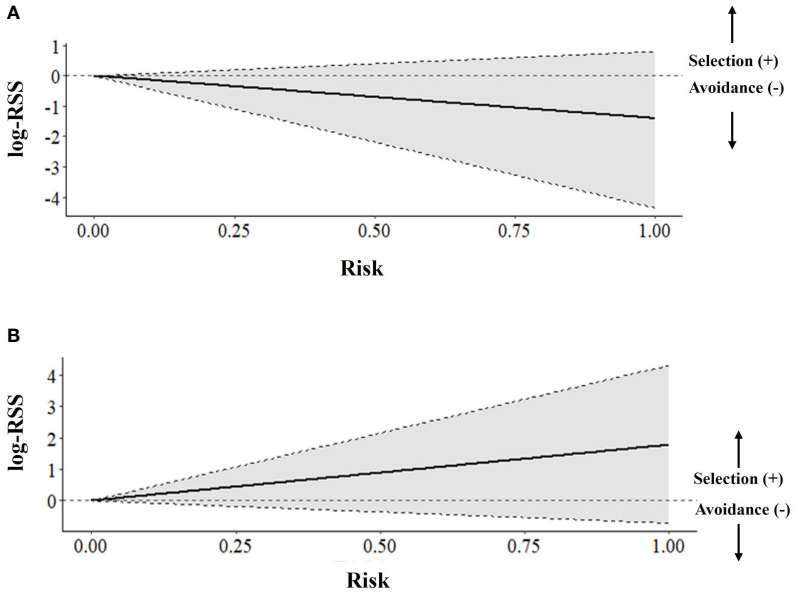

FIGURE 7
Selection and avoidance of areas at higher risk of mortality for Greater sage-grouse (*Centrocercus urophasianus*) that were captively bred and released in **(A)** Alberta and **(B)** Saskatchewan, Canada (2018 -2022), as assessed by an integrated step-selection function analysis. Plots depict population-level estimates of log-relative selection strength (log-RSS) obtained via inverse-variance-weighted regression on individual models, using the risk surface layer.

Aldridge and Boyce, 2007; Cutting et al., 2019; Lazenby et al., 2020; Pratt and Beck, 2021). Wild birds can select familiar places (e.g., nesting sites) that have lower nest or chick survival (Aldridge and Boyce, 2007), resulting in population sources and sinks (Heinrichs et al., 2018). Accordingly, the movement selection results for Saskatchewan indicated that released grouse selected risky areas. Their preferences for resource-rich sites may have outweighed their perceived risk of predation. This inconsistency in released birds' movement and selection behaviors among release areas emphasizes the value of building models for unique environmental contexts. When sufficient data becomes available to assess mortality risks in specific areas, we recommend updating model extrapolations from other areas with site-specific models.

We lacked sufficient data to fully assess mortality risk using step selection functions. However, we found this multistep approach to be a useful coupling of methods to assess congruence of survival with movement selection and avoidance; ultimately, resulting in evidence that released individuals are making at least some choices that optimize their fitness, and some that are not. We used daily sage-grouse locations, but with increased temporal resolution of the data, this analysis could be expanded to assess finer-scale movement behavior that may further illuminate risks and movement responses associated with specific environmental features.

4.3 Predation and environmental factors

Coyotes and raptors, such as the great horned owls, are the apex predators of this ecosystem (Ritchie and Johnson, 2009) and use anthropogenic infrastructure to move and hunt. We lacked data on predator abundance and distribution and were unable to directly assess risk factors associated with predation; however, as most sage-grouse deaths were caused by predation, we infer mortality risk is primarily driven by predation and factors that facilitate predation. High mortalities in the presence and absence of anthropogenic infrastructure (in Alberta and Saskatchewan, respectively) indicate there is more to learn about the causes of mortalities in each place, including predator communities and the environments that facilitate predation. This risk assessment could be augmented with maps that describe predation pressure and predator abundance and distribution results from long-term systematic surveys. Mortality risk maps (e.g., Figures 5A, 6A; Supplementary Figures 3, 4) could be used to support the design of predator surveys and utilization maps to spatially target areas in which to deter predator use (e.g., through removal of attractants; Parks Canada Agency, 2021). This may be particularly useful for canid predators, such as coyotes, that are opportunistic feeders and able to exploit a variety of environments and resources (Bartel and Knowlton, 2005).

Further analyses of predator data can be strengthened with molecular analyses to improve predator determination (Peelle et al., 2019); however, preliminary analyses suggest that canids, corvids (e.g., American crows, *Corvus brachyrynchos*; common raven, *Corvus corax*) and owls (e.g., great horned owl) (Quinlan, 2013) are key consumers of released sage-grouse. The expansion of corvid surveys in Alberta to include a broader range of predators would help researchers and managers better understand sage-grouse mortality risk. Future analyses could also consider how the landscape facilitates and protects sage-grouse from weather exposure during extreme weather situations, as well as incorporate additional risk factors as data become available (e.g., smaller built features such as power poles, transmission lines, fences).

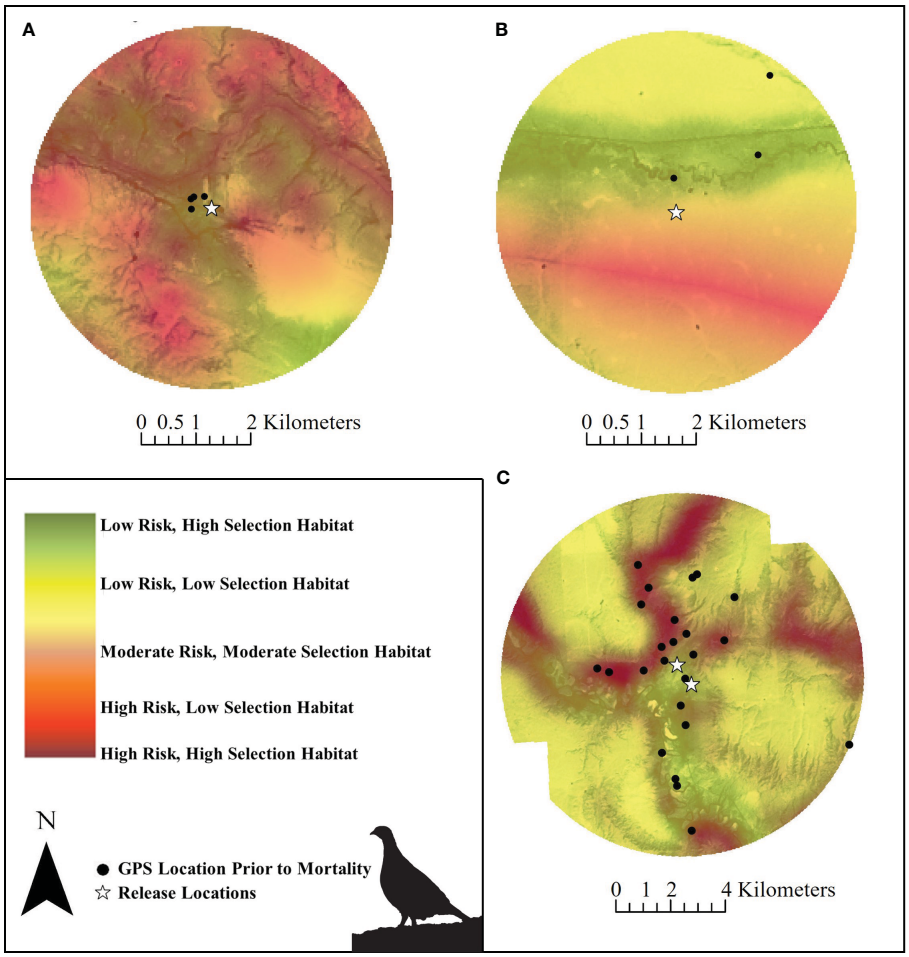

FIGURE 8
Mortality risk associated with different levels (e.g., low, high) of habitat selection near Greater sage-grouse release sites in Alberta and Saskatchewan, Canada (2018 – 2022). Mortality risk was influenced by anthropogenic features as well as habitat (continuous critical habitat; Environment Canada, 2014) and displayed on the critical habitat map to highlight areas where habitat selection and mortality risk (mis)align at release sites in Alberta **(A)** northwest, **(B)** southeast, and **(C)** Saskatchewan release sites. Silhouette of Greater sage-grouse © Cornell Lab of Ornithology.

4.4 Non-spatial risk factors

Beyond environmental associations of mortality risk, we also examined the factors associated with release including age and weight at release, acclimation period, raising method, release period (e.g., release before October 17, or release after October 17), and release type (e.g., hard, soft, and modified-soft release). Our results suggest the spatial-environmental factors that we measured were generally more influential than factors related to the release protocol in affecting overall mortality risk. However, we did find that some of these protocol-related factors were explanatory, depending on how the data were analyzed, for which we sought alternative ways to analyze the data (see Supplementary Material for alternative models). For example, if we looked at mortality outcomes by season, the model fit was not as strong, but we occasionally found a signal of age and weight at release (only in Saskatchewan during the fall; Supplementary Figure 2B).

Although the influence of animal traits and release protocol factors were minor relative to the release environment, there could be other unquantified conditions that influence mortality including animal health (e.g., stress), predator avoidance conditioning, and diet adaptation. For example, some birds can die earlier due to health or disease-related issues and (or) because of lack of environmental conditioning (e.g., diet is the ultimate cause, and predation is the proximate cause). Captive breeding and translocation programs can impose stress on animals during captivity, handling, transportation, and release experiences (Dickens et al., 2010). Stress can influence behavior (e.g., risk taking; May et al., 2016) including movement and habitat selection (Osborne and Seddon, 2012), and reduce survival of reintroduced individuals (Bremner-Harrison et al., 2004). Stress and disorientation induced by the release can lead individuals to select less suitable areas outside of strategically chosen release sites with suitable habitat. Individuals may have further difficulties in transitioning between supplementary feed and natural diets due to their reliance on supplementary feeding (Draycott et al., 1998). Survival times are generally longer for wild-to-wild sage-grouse translocations in Alberta, with average hen survival (n = 77 with

known fates) of close to a year (296 days, and some survived >1 year; unpublished data), suggesting that there are likely to be some unquantified factors beyond environmental features that are important to the survival of released sage-grouse. As these factors are quantified, they can be used to enrich this analysis.

4.5 Conservation implications

The results of this analysis can support adaptive planning and management of the release program and environment. The success of captive breeding and translocation programs depends not only on the quantity and quality of animals released, but also on identifying and managing the factors that contribute to their survival in the wild (Berger-Tal et al., 2020). Some of the elements associated with post-release mortality can be managed, reduced, or mitigated. Beyond optimizing captive and release protocols, the environment into which the animals are released should also be managed to reduce mortality risk (IUCN/SSC, 2013). For example, mortality risks for Galliformes can be partially mitigated by selecting release locations and surrounding areas that minimize opportunities for predation or predation pressure (Warren and Baines, 2018), and maximize food and cover resources. Future release sites and surrounding areas in Alberta and Saskatchewan can be screened using the risk map or associated spatial data layers to further optimize site selection. Sage-grouse release areas differed in their habitat characteristics and mortality risks. Thus, future release sites could be chosen to balance the risk profile of existing sites by releasing animals into areas with lower overall risk and a different balance of risk factors. This cycle of adaptive management can benefit from strong collaborations among translocation professionals and data scientists that can make use of small datasets, as well as species and land managers and stewards that can put results into action.

Actions are being taken by a range of government, conservation, and industry groups to reduce anthropogenic infrastructure in Canada and benefit sage-grouse. Results suggest the removal of oil and gas infrastructure, and predator perches including trees, buildings, and other vertical features (Whiklo and Nicholson, 2015) will change the spatial distribution of mortality risk, and these actions have the potential to reduce mortality risk for released sage-grouse. However, it is unclear whether infrastructure removal by itself would be sufficient to sustain released sage-grouse. As wild populations have been declining for decades in Canada, it is likely that several factors are contributing to high mortality rates, and several kinds of population recovery and habitat restoration actions are needed to stabilize the population trajectory. Although some threats sage-grouse face cannot be directly managed (e.g., climate patterns, severe weather events), a number of threats can be mitigated indirectly through habitat improvement such as encouraging natural hydrology in mesic areas (e.g., constructing ditch or dam) to augment sage-grouse forage (Alberta Environment and Sustainable Resource Devlopment, 2013; Environment Canada, 2014). Models that link spatial mortality risk to landscape projections could be used to scope the population gains associated with complementary actions that benefit both wild and released sage-grouse (Heinrichs et al., 2018, Heinrichs et al., 2019).

4.6 Doing more with less data

We used a multiphase approach to assess the environmental factors influencing mortality risk, whether existing habitat maps are likely to predict where released animals are likely to survive, and the degree to which animals' movements corresponded to risk correlates. We implemented a workflow that addresses the challenge of having few surviving animals and small location datasets, a situation common in captive breeding and release programs. We suggest this linkage of analyses is helpful for several reasons. First, it yielded a simple yet actionable risk map that complements existing information on habitat, populations, and landscape features. Viewing habitat and mortality risk in the same map highlighted locations where the conditions are appropriate for both habitat selection and mortality, and locations where landscape improvement actions may yield high returns on ecological investments. Second, this workflow was also useful in evaluating the assumption that 'good' habitat in habitat selection maps represent optimal places in which to release translocated animals. We found several locations where highly selected habitat is associated with a high risk of mortality. Mortality risk maps can complement existing habitat maps by including additional factors that were excluded from the habitat map that may not have a strong signal of selection but influence survival. Our mortality model included anthropogenic and other features that facilitate predation that were not included in the habitat model. Mortality models could additionally include predator locations, densities, preferences, or locations that lack resources to enable animals to withstand extreme weather. Lastly, this workflow evaluated movement behavior relative to the risk landscape and provided a means to assess whether animals are responding to risk features. Movement and selection responses were different between release areas (Alberta and Saskatchewan), indicating that release success, and the prescriptions to enhance post-release survival, may differ among locations. Actions that address high mortality in high selection areas may be more influential than those in low selection areas (e.g., Heinrichs et al., 2018).

Although we found a path to mapping post-release mortality risk that led to key management insights, this approach had limitations. For instance, we used a small dataset where seasons, sexes, and ages were combined, and environmental covariates were combined in composite layers. Consequently, we may have missed relationships that could be found in larger datasets, including those related to release protocols and interacting environmental conditions. A larger dataset would have streamlined the workflow by allowing us to jointly evaluate several environmental covariates in a single model, as well as investigate differences among individuals (e.g., traits, status), differences in release protocol, and differences among behavioral and movement states (e.g., encamped,

exploring, travelling, relocating; Harju et al., 2013; Picardi et al., 2021). Despite these limitations, we found empirical patterns that can be used to support near-term conservation actions while additional data are collected and used to develop the next generation of risk models. We used concepts and tools that most wildlife researchers are familiar with, using free and accessible software packages. As such, we suggest this workflow may be useful for other wildlife researchers facing similar constraints and may be particularly useful in cases where mismatches in habitat selection and survival are likely to occur.

5 Conclusion

As the biodiversity crisis intensifies (Butchart et al., 2010), conservation practitioners will increasingly need to make high-stakes decisions with analyses that make adaptive use of available information (Berger-Tal et al., 2020). Captive breeding and release programs exemplify this situation. Translocation planners need to make decisions on where to release animals and how to modify the release environment at the onset of the program with information that is on-hand. Data from extant wild populations and associated habitat maps can provide insight into the environmental factors that are generally associated with a species' needs or preferences; however, they can paint an incomplete picture of the movements, behaviors, and survival of captively-bred and released animals. This Galliform case study highlights the potential for habitat to be a partially misleading indicator of optimal release sites and post-release survival. This underscores the value of assessing the mortality risks associated with the release environment. As many factors shape survival in the post-release landscape, it can be challenging to identify the factors that are most important to understand and manage. Spatial risk analyses can help direct attention to key environmental factors that may be limiting translocation success, structure adaptive changes to both release protocols and the release environment, and support conservation teams to overcome disruptions in the adaptive management cycle.

Data availability statement

Data cannot be openly shared due to sensitive location information. However, further inquiries regarding data accessibility can be directed to the Wilder Institute/Calgary Zoo. Requests to access these datasets should be directed to MillieC@calgaryzoo.com.

Ethics statement

The animal study was approved by Animal Welfare, Ethics and Research Committee (AWERC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SN: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. JS: Writing – review & editing, Visualization, Software, Investigation. JH: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. DM: Writing – review & editing, Supervision, Project administration, Methodology, Data curation, Conceptualization. AC: Writing – review & editing, Supervision, Project administration, Methodology, Data curation, Conceptualization. IG: Writing – review & editing, Supervision, Project administration, Methodology, Data curation. TW: Writing – review & editing, Methodology, Conceptualization. AM: Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. JAH: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

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(Todd Whiklo). Grasslands National Park and The Nature Conservancy of Canada allowed the construction of acclimation pens on their property. Identification of predators based on remains collected at predation sites was conducted by Alberta Environment and Protected Areas (Gordon Court) and Wilder Institute/Calgary Zoo (Dario Fernandez-Bellon).

Conflict of interest

Authors SN, JS, JH, and JAH were employed by the company Computational Ecology Group Inc.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2024.1393264/full#supplementary-material

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Hormone therapy improves conservation breeding outcomes in the critically endangered Baw Baw frog, *Philoria frosti*

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Conservation breeding programs (CBPs) are often the lifeline between extinction and survival for many imperilled amphibian species. With the goal of recovering wild populations, CBP success is reliant on their ability to successfully manage *ex situ* populations over time, breed viable offspring, and maintain genetic diversity and adaptive potential. Reproductive technologies have emerged as an important tool in the conservation toolkit to allow managers to improve reproductive output and genetic management, and their use in amphibian conservation is expanding. To date, studies investigating the efficacy of hormone therapies in amphibians typically only report spawning and fertility rates and do not monitor offspring to later stages of development. For the first time, here we assess the effect of hormone therapies on captive breeding outcomes beyond oviposition, to the point of metamorphosis, in the critically endangered Baw Baw frog, *Philoria frosti*. To determine the effect of hormone therapy on spawning success and offspring viability, male-female pairs were administered either 0 µg/g gonadotropin-releasing hormone agonist (GnRHa), 0.5 µg/g GnRHa, or 0.5 µg/g GnRHa + 10 µg/g metoclopramide (MET) (n = 12 pairs/treatment), and the number of pairs ovipositing, time to oviposition, clutch size, metamorph mass, and the proportion and number (mean and total) of offspring to metamorphosis were quantified. Overall, the percentage of pairs that oviposited was high across all treatment groups (92-100%). The percentage of fertile clutches was highest in the GnRHa group (92%) and lowest in the GnRHa + MET group (82%), though differences were not statistically significant. Both hormone treatment groups took significantly less time to oviposit than the control pairs. Notably, the proportion of eggs developing to metamorphosis was significantly higher in the GnRHa group, resulting in 74% (total eggs=539) metamorphosing compared to approximately 50% in the control and GnRHa +MET treatments (total eggs= 273 and 264, respectively). Interestingly, weight at metamorphosis was statistically similar across all groups, and results are consistent with previous studies in this species that show a narrow range in size at metamorphosis. The continued application of GnRHa is recommended to

improve conservation outcomes for the critically endangered Baw Baw frog. The outcomes of this research advance our understanding of the impact of hormone therapies on reproductive outcomes and will inform amphibian conservation breeding programs globally.

KEYWORDS

amphibian, captive breeding, conservation, gamete-release, oviposition, offspring, reproductive technologies, hormone therapy

1 Introduction

Conservation breeding programs (CBPs) are increasingly vital in mitigating biodiversity loss by safeguarding threatened species (Stuart et al., 2004). These programs serve as a crucial measure to prevent species extinction and provide a buffer period for effectively managing key threatening processes. Typically, CBPs engage in addressing multiple objectives, including preserving genetic diversity, breeding for population recovery (supplementation, introduction, or reintroduction), conducting targeted research on threats, or a combination of these (Mcfadden et al., 2018).

While the decline of vertebrate taxa has reached unprecedented levels, amphibians are in the most perilous position. An estimated 41% of the 8,020 assessed amphibian species are currently threatened with extinction (IUCN, 2023), making them a focal taxon for the development of CBPs. Compared with many other taxa, amphibians are typically smaller in body size, meaning that their long term management under *ex situ* CBPs demands less infrastructure, resources, and financial investment (Stuart et al., 2004). However, effective establishment of amphibian CBPs is often marred as a result of delayed initiation of programs until population sizes become critically low, and/or limited knowledge of a species' captive requirements (Harding et al., 2016). Couple this with the fact that many species display complex life-histories and require specific environmental cues to stimulate breeding, research to refine conservation breeding approaches is critically needed. In particular, protocols to optimize reproduction, growth, and development are urgently needed to ensure the retention of genetic diversity and facilitate long-term program success.

Within the conservation management tools available for CBPs, reproductive technologies such as hormone therapies, assisted fertilization, and genome resource banking, are increasingly being employed to assist with reproductive output and genetic management (Browne et al., 2019; Clulow et al., 2019; Silla et al., 2023; Silla and Kouba, 2022; Della Togna et al., 2020). These technologies have been applied widely across mammalian taxa (Holt, 2003) but are now increasingly being developed and applied to amphibians. Reproductive technologies allow conservation managers to improve propagation (breeding participation, spawning success, clutch size), facilitate pair-wise breeding (control genetic couplings), improve fertilization rates, and enhance the genetic diversity of offspring (Silla and Byrne, 2019). While the utilization of reproductive technologies will no doubt provide valuable management strategies for optimizing amphibian CBPs and assisting threatened species recovery, adoption of these methods remains limited to only a small proportion of total threatened species. This is in part because the successful application of reproductive technologies requires species-specific optimization and refinement to produce effective results (Silla and Byrne, 2019). Such species specificity makes the development of broad treatment protocols challenging. Equally challenging for conservation managers is a current gap in knowledge regarding the assessment of fitness determining traits of offspring generated through the application of reproductive technologies (Silla and Byrne, 2024). Specifically, often studies reporting on the development of reproductive technologies only assess the number of clutches oviposited or fertility rates and typically do not monitor offspring throughout development, particularly to critical life stages such as metamorphosis.

The critically endangered Baw Baw frog, *Philoria frosti*, is endemic to Mount Baw Baw plateau in Victoria, Australia (Hollis, 2011). Once abundant, the species has suffered catastrophic wild population decline and is now perilously close to extinction in the wild (D.J. Gilbert, pers. comm.). While factors contributing to the decline are not completely understood, most evidence suggests that the declines are linked to lethal effects of the chytrid fungus (*Batrachochytrium dendrobatidis*) (Burns, 2021; Hunter et al., 2018). Baw Baw frog survival currently relies on a dedicated CBP that broadly aims to; i) optimize growth and development across all life stages (Gilbert et al., 2020), ii) optimize breeding and translocation protocols, and iii) develop effective biobanking tools to preserve genetic diversity (Silla et al., 2023). Here we aimed to build on reproductive technologies previously developed for this species (see Silla et al., 2023) by testing the efficacy of hormone therapy on spawning success in pair-wise (male-female) treatment groups. Male-female pairs were allocated to one of three treatments: 1) a control treatment with no hormone therapy, 2) an experimental treatment whereby frogs were treated with gonadotropin releasing-hormone analogue (GnRHa), and 3) an experimental treatment whereby frogs were treated with GnRHa plus metoclopramide. For the first time we follow developing offspring through to metamorphosis, providing results of the effect of hormone therapies on the proportion and total eggs developing to

metamorphosis and the size of offspring at the time of metamorphosis.

2 Materials and methods

2.1 Ethics statement

The protocols and procedures described herein were conducted following review and approval by the Zoos Victoria Animal Ethics Committee (ZV22009) in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes.

2.2 Study species

The Baw Baw frog is a medium-sized (46-55mm snout-vent length) terrestrial species with a large parotoid gland positioned on each shoulder (Figure 1A). Having disappeared from much of its former range, the species is now restricted to a small area of protected montane gully habitat (1000-1300m altitude), on the Mount Baw Baw Plateau in the Central Highlands of Victoria, Australia (Gilbert et al., 2020). The Baw Baw frog is a long-lived species with an estimated lifespan of 17+ years, males are known to reach sexual maturity at 3.5 years and females at 4.5-5.5 years (Hollis, 2011). Commencement of the annual breeding season corresponds with an increase in ambient temperature during austral spring (Hollis, 2011). Male calling behavior occurs from September to March, with a peak in October and November which coincides with peak breeding activity (Hollis, 2011). Breeding typically occurs along shallow seepage lines, with oviposition taking place below the surface within natural cavities formed from vegetation, fallen logs, and embedded rocks (Clemann and Swan, 2023). Terrestrial nest-sites vary in their depth below ground, and while wet, typically retain little free water (Hollis, 2011; Gilbert et al., 2020). Amplexus in this species is inguinal and relatively large, unpigmented eggs are deposited into a foam nest (Hollis, 2011) (Figures 1B, C). Hatching has been observed to occur between 10-15 days following oviposition (D. Gilbert unpublished data). Tadpoles hatch and develop within the nest site, or may be free-swimming if washed into shallow waterbodies nearby (Clemann and Swan, 2023; Gilbert et al., 2020). Under natural field conditions, tadpole development to metamorphosis is completed within 10-18 weeks (Malone, 1985).

2.3 Study animals and adult husbandry

A total of 36 adult male and 36 adult female Baw Baw frogs were involved in the study, representing the current adult breeding stock of the conservation breeding and reintroduction program for the species. All frogs were founding animals either collected from the wild as adult individuals, or reared in captivity from wild collected clutches, obtained from natural populations located at Mount Baw Baw, Victoria. Ages of both the male and female frogs ranged between approximately nine and sixteen years old. Baw Baw frogs were maintained in two isolated biosecurity facilities located at Zoos Victoria's Melbourne Zoo (Parkville, VIC, Australia). Internal lighting within the facilities were controlled using a photocell light-sensitive sensor set to replicate local photoperiod. During the experimental period, lighting was provided using LED plant spectrum tubes (Fluval 3.0 Plant Spectrum) suspended above each shelf and programmed to provide varying color spectrum and intensity throughout the day. Ambient temperature within the facilities is cycled annually to reflect seasonal changes in the average climatic conditions experienced in the alpine areas on Mount Baw Baw where the species naturally occurs. Annual temperatures range from 5 to 15°C, including a 6-week overwintering period (21st July- 31st August). Frogs entering the breeding tanks at the commencement of the study were maintained on a 10°C/15°C night/day temperature cycle, corresponding with the natural conditions experienced during peak breeding activity in the field. The frogs were fed twice per week alternating between medium sized crickets (*Acheta domestica*; ~3-5 crickets per individual) and pill bugs (*Armadillidium vulgare;* ~3-5 pill bugs per individual). Crickets were gut-loaded 48 hours prior to feeding with insect booster (Womberoo) and dusted with a multivitamin supplement (Multical dust, Vetafarm) prior to feeding. Pill bugs were fed without supplementation due to their naturally high calcium levels.

2.4 Hormone-induced spawning

To determine the effect of hormone therapy on spawning success, 36 male-female pairs were allocated to one of three treatments (n = 12 pairs per treatment): 1) 0 µg/g GnRHa (control group), 2) 0.5 µg/g GnRHa, and 3) 0.5 µg/g GnRHa + 10 µg/g metoclopramide (MET; Sigma-Aldrich). The hormones selected and their formulations were chosen based on a

FIGURE 1
Baw Baw frog, *Philoria frosti*. Images shown are **(A)** an adult male Baw Baw frog, **(B)** male-female pair in amplexus, and **(C)** amplectant pair spawning unpigmented embryos into a foam nest. Photographs courtesy of Damian Goodall- Zoos Victoria.

preliminary study in this species (Silla et al., 2023), in addition to previous research in other anuran species (Trudeau et al., 2013, Trudeau et al., 2010). In brief, GnRHa is one of the most commonly employed exogenous hormones to induce spawning and gamete-release in amphibians, with recent research into the use of GnRHa in combination with MET (or other dopamine antagonist) as a possible way of potentiating the effect of GnRHa (reviewed in Silla and Langhorne, 2022). The GnRH analogue (leuprolide acetate salt; Sigma-Aldrich) was suspended in bacteriostatic saline (Bacteriostatic Water Australia) to generate a 5mg/ml stock suspension prior to further dilution to the required dose. Hormones were diluted in 100 µL of simplified amphibian ringer (SAR; composition (in mM): NaCl 113; KCl 2; $CaCl_2$ 1.35; $NaHCO_3$ 1.2) and administered via subcutaneous injection into the dorsal lymph sac using ultra-fine 31-guage needles, following hormone injection protocols used previously (Silla et al., 2023, Silla et al., 2019). Frogs in the control treatment received 100 µL of simplified amphibian ringer only.

Individuals within each male-female pair were administered a single hormone injection corresponding to their experimental treatment. Hormone administration occurred directly prior to each pair entering the breeding tanks. Males and females within each pair were injected at the same time, as previous research has shown that this approach is more effective than administering hormones to male frogs prior to females (Silla et al., 2018). Immediately prior to hormone injection, frogs were weighed to the nearest 0.01 g and the dose administered was adjusted according to an individual's body mass. Male body mass ranged from 9.29 g to 16.39 g (mean± SEM male mass = 12.05 ± 0.27 g; n=36). Female body mass ranged from 12.77 g to 23.46 g (mean± SEM male mass = 17.01 ± 0.42 g; n=36). The body mass of males and females did not differ significantly between treatment groups (one-way analysis of variance (ANOVA), male mass: $F_{2,35} = 0.303$, $P = 0.740$; female mass: $F_{2,35} = 2.325$, $P = 0.114$).

Following hormone administration, male-female pairs were placed into specifically designed breeding enclosures, one pair per enclosure (Figures 2A, B). Each breeding enclosure (36cm x 60cm x 24.6cm) consisted of a glass aquarium with a raised, ventilated mesh canopy (36cm x 60cm x 35cm). Each enclosure was connected to an automated irrigation system circulating carbon filtered water. To offer a variety of potential oviposition sites, each enclosure contained a layer of aquarium gravel, which varied in depth throughout the enclosure to create shallow pools, established live plants, and pieces of curved plastic (Figures 2A, B). Following hormone treatment, breeding enclosures were visually inspected daily for the presence of eggs and the date of oviposition recorded. Unfertilized eggs or those exhibiting early embryonic failure were carefully removed from the clutch to avoid decomposing eggs negatively influencing viable embryos (Silla and Byrne, 2024). Male-female pairs entered the breeding enclosures on October 10, 2023, during the peak of the species' natural breeding season.

2.5 Offspring husbandry

Eggs and tadpoles were reared in the same two isolated biosecurity facilities where the adult breeding enclosures were located. Eggs were removed from the breeding tanks approximately 2 days following oviposition to minimize disturbance from the adults and placed into tadpoles rearing tanks. Throughout egg and tadpole development, offspring were maintained on a 12°C/15°C night/day temperature cycle. Tadpole rearing tanks were constructed with purpose-built aquaria arranged in banks, each containing 10 individual tanks measuring 21cm x 22cm x 20cm. Two banks were constructed totaling 20 individual tadpole tanks, each clutch was reared individually. These tanks were divided by mesh screens to allow for air and water movement. Additionally, each bank, measuring six tanks long and two tanks wide, featured a sump area (16cm x 22cm x 20cm) at one end equipped with an aquarium water pump to facilitate water flow. An overflow system was incorporated for water change filtration. To simulate natural habitat conditions, approximately 3cm of substrate, composed of decomposing granite and organic material sourced from field breeding sites, was added to the bottom of each tadpole tank. To mitigate the risk of disease transfer, the substrate underwent heat treatment and drying (40°C for 12 hours) before being introduced into the tanks. Under natural

FIGURE 2
Captive breeding facility and metamorph frogs. Images shown are **(A, B)** Baw Baw frog breeding enclosures with established natural substrate and automated irrigation and filtration, and **(C)** Baw Baw frog offspring generated from the present study following release to wild habitat at Mount Baw Baw. Photographs courtesy of Damian Goodall and Deon Gilbert- Zoos Victoria.

conditions Baw Baw frog larvae develop in darkness in underground cavities and are sensitive to light during development (Hollis, 2004). To ensure minimal light disturbance in the captive facility, the sides of the tanks were covered with black vinyl contact, and a removable black plastic lid was fitted on top. Tadpoles were monitored approximately once per week to track development progress and remove any dead or rotting eggs. Water depth in each tank was maintained at approximately 2cm, and the internal substrate was graded on an angle to create a small terrestrial portion, thus preventing drowning during metamorphosis. All offspring metamorphosed between December 10-16, 2023, approximately 9-weeks after the date of oviposition. Following complete metamorphosis (full tail reabsorption), a subset of frogs were placed on a small (2.5 cm diameter) petri dish and weighed to the nearest 0.01 g using digital scales (Pesola touch screen digital pocket scale). Overall, 51 metamorphs were randomly selected from 9 clutches to be weighed, a total of 17 individual offspring from each of the three treatment groups, control, GnRHa and GnRHa + MET. To supplement the assurance colony, 7-11 individuals from 17 clutches (168 offspring in total) were retained at Melbourne Zoo. All 709 remaining offspring were released back into the wild on Mount Baw Baw to augment natural populations for the species (Figure 2C).

2.6 Statistical analyses

The number of male-female pairs ovipositing, and the number of fertile clutches laid were compared between treatment groups using ChiSquare Likelihood Ratio Tests. One-way ANOVAs were used to test for statistical differences among treatment means in; 1) days until oviposition, 2) clutch size, 3) metamorph mass, 4) number of individuals to metamorphosis, and 5) proportion to metamorphosis. Comparisons among treatment means were conducted using each pair student's t *post hoc* tests. Prior to ANOVA analysis, to verify homogeneity of variances, Browne-Forsythe equivalence tests were performed. For all response variables variances were equal ($P > 0.05$) and data were not transformed prior to analysis. All statistical analyses were performed using JMP Pro 16.1.0 software package (SAS Institute Inc.) and statistical significance was accepted at $P < 0.05$.

3 Results

Overall spawning success was high across all experimental treatments, with 92%-100% of male-female pairs ovipositing (Table 1). Of the pairs that successfully oviposited, the percentage of fertile clutches (those producing fertilized embryos) was highest in response to the administration of GnRHa (92%), though differences between treatment groups were not statistically significant ($\chi^2 = 0.580$, $p = 0.748$; Table 1). Hormone treatment had a significant effect on the time taken for pairs to oviposit a clutch of eggs (ANOVA, F-ratio $_{2,34} = 4.142$, $p = 0.025$). Following hormone administration, pairs in the GnRHa and GnRHa + MET treatment groups took a significantly shorter time to oviposit compared to pairs within the control treatment (each pair student's t *post hoc* tests, $p < 0.05$), which, on average, took an additional 3 days (Table 1). Pairs that oviposited released between 30-80 eggs, with a larger mean clutch size oviposited in response to the administration of GnRHa compared to the GnRHa + MET treatment (each pair student's t *post hoc* tests, $p < 0.05$; Table 1). The mean clutch size released by pairs in the control treatment was not significantly different to either of the hormone treatments (each pair student's t *post hoc* tests, $p > 0.05$; Table 1).

Of the subset of juveniles that were weighed (n=17 per treatment), frog mass at the time of metamorphosis ranged from 0.05 to 0.12 grams. Mean metamorph mass was statistically similar across the three treatments groups (ANOVA, F-ratio $_{2,50} = 1.747$, $p = 0.185$; Table 1). Hormone treatment had a significant effect on

TABLE 1 The effect of hormone treatment on spawning and offspring viability.

Response variable	Hormone treatment			*P-value*
	0 µg/g GnRHa (control)	0.5 µg/g GnRHa	0.5 µg/g GnRHa + 10 µg/g MET	
Pairs ovipositing (%)	12/12 (100%) ^{A}	12/12 (100%) ^{A}	11/12 (92%) ^{A}	0.324
Fertile clutches (%)	10/12 (83%) ^{A}	11/12 (92%) ^{A}	9/11 (82%) ^{A}	*0.748*
Days until oviposition	9.8 ± 4.67 ^{A}	6.42 ± 1.98 ^{B}	6.64 ± 2.29 ^{B}	*0.025* *
Clutch size (number of eggs)	49.58 ± 4.71 ^{AB}	60.83 ± 4.35 ^{A}	45.00 ± 5.57 ^{B}	*0.078*
Metamorph mass (grams)	0.082 ± 0.004 ^{A}	0.092 ± 0.004 ^{A}	0.085 ± 0.004 ^{A}	*0.185*
Mean number per clutch to metamorphosis	22.75 ± 7.04 ^{B}	44.92 ± 6.96 ^{A}	23.67 ± 5.94 ^{B}	*0.040* *
Proportion per clutch to metamorphosis	37.31% ^{B}	69.65% ^{A}	48.48% ^{AB}	*0.048* *
Total offspring per treatment to metamorphosis	273 (54%)	539 (74%)	264 (49%)	-

Data shown are the number of pairs ovipositing/total number of pairs (%), number of fertilie clutches oviposited/total number of pairs (%), or mean ± SEM (days until oviposition, clutch size, metamorph mass, number to metamorphosis, proportion to metamorphosis). For all analyses sample sizes were 12 replicates per treatment, with the exception of mass at metamorphosis where a subsample of 17 individuals per treatment from 9 clutches were analysed. Data were analysed using ChiSquare Likelihood Ratio Tests (pairs ovipositing, fertilie clutches), or one-way ANOVAs (days until oviposition, clutch size, metamorph mass, number to metamorphosis, proportion to metamorphosis). * denotes a significant P-value. Letters displayed are the result of post-hoc tests (each pair student's t). Within a row, treatments that share a letter are not significantly different (P>0.05). See methods for details of all statistical analyses.

the mean number of offspring per clutch that developed to metamorphosis (ANOVA, F-ratio $_{2,34}$ = 3.542, p = 0.040), which was significantly higher in the GnRHa treatment compared to the two remaining treatments (each pair student's t *post hoc* tests, p<0.05; Table 1). Similarly, the proportion of offspring that developed to metamorphosis was significantly different among treatment groups (ANOVA, F-ratio $_{2,34}$ = 3.356, p = 0.048). The mean proportion of offspring to metamorphosis from each clutch ranged from 37.3% to 69.7%, with the highest percent exhibited from clutches laid in response to the administration of GnRHa (Table 1). Overall, the cumulative total number of offspring that developed to metamorphosis was 539 (74%) for the GnRHa, compared with 273, (54%) and 264 (49%), in the control and GnRHa + MET treatments, respectively (Table 1).

4 Discussion

The Baw Baw frog wild population is perilously close to extinction and without a dedicated CBP capable of producing high numbers of viable, genetically representative, offspring for translocation the chance of wild recovery will be low. Optimizing reproductive output and effective genetic management are key to the long-term sustainability of the CBP, hence developing tools to ensure these goals are met is critical. Reproductive technologies have emerged as a potential management tool to increase both reproductive output and genetic diversity by providing animals with exogenous hormones to stimulate reproductive events, including spawning (e.g. see Silla et al., 2018). The aim of this study was to build on hormone therapy protocols previously developed for this species (Silla et al., 2023), to test the effect of GnRHa or GnRHa + MET on spawning success in male-female pairs, and on the viability of offspring up until the point of metamorphosis.

Results showed that spawning success was high across all treatment groups. Overall, the percentage of fertile clutches oviposited was highest in pairs administered GnRHa (92%), and lowest in pairs administered GnRHa + MET (82%), though differences between treatment groups were not statistically significant. Both hormone treatment groups (GnRHa and GnRHa + MET) took significantly less time to oviposit compared to the control pairs, which on average took an additional three and a half days to oviposit. The administration of GnRHa has been used to successfully induce spawning in male-female pairs/groups, as well as gamete release in isolated animals, in a diversity of amphibian species (Uteshev et al., 2013; Sherman et al., 2008; Jacobs et al., 2016; Otero et al., 2023; Silla and Byrne, 2021; Silla et al., 2018; Silla, 2011; Guy et al., 2020). The administration of GnRHa mimics natural GnRH-1 molecules, binding to receptors on the anterior pituitary to stimulate the endogenous synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Vu and Trudeau, 2016). Over the past decade, there has been growing interest in the administration of GnRHa in combination with a dopamine antagonist (domperidone, pimozide, or metoclopramide), which is hypothesized to attenuate the stimulatory effect of GnRHa by limiting dopaminergic inhibition (Vu and Trudeau, 2016). The combined administration of GnRHa plus metoclopramide, a method also referred to as AMPHIPLEX, has been successfully used to induce spawning in a number of species, including northern leopard frogs, tiger frogs, Argentine horned frogs, Cranwell's horned frogs, and common lesser escuercito (Trudeau et al., 2010; Godome et al., 2021). However, studies on the northern leopard frog, American bullfrog, and Panamanian golden frog report statistically similar spawning rates in frogs administered GnRHa + MET compared with GnRHa alone at an optimal dose (Bronson et al., 2021; Nascimento et al., 2015; Vu et al., 2017). Our previous research inducing spawning in the Baw Baw frog, reported no statistical difference in the spawning success of pairs administered GnRHa compared to pairs receiving GnRHa + MET, though a higher proportion of pairs in the GnRHa + MET treatment oviposited (Silla et al., 2023). In the present study, sample sizes were almost doubled and, consistent with research in the northern leopard frog, American bullfrog, and Panamanian golden frog (Bronson et al., 2021; Nascimento et al., 2015; Vu et al., 2017), our data show no difference in spawning rates between hormone treatments.

While a growing number of studies are investigating the use of hormone therapies to induce spawning and improve captive-breeding outcomes for amphibians, there remains a lack of data quantifying outcomes beyond oviposition and fertilization. As such, there is limited understanding of the degree to which specific protocols might influence (either improve, or potentially compromise) offspring fitness (such as body size and survival rates between developmental stages) and lead to shifts in population viability (Silla and Byrne, 2024). For the first time, we monitored offspring throughout development to the point of metamorphosis, across each treatment group. Our results demonstrate that significantly more offspring developed to metamorphosis in the GnRHa treatment group compared to the control or GnRHa + MET treatments. Both the mean number of offspring metamorphosing, and the proportion of offspring developing to metamorphosis was significantly higher in the GnRHa group. The GnRHa treatment resulted in 74% of eggs metamorphosing (total eggs=539), compared to approximately 50% in the control and GnRHa + MET treatments (total eggs = 273 and 264, respectively). Additional research will now be required to determine the mechanism by which the addition of MET resulted in fewer offspring reaching metamorphosis compared to GnRHa alone. Overall, the administration of GnRHa resulted in a 25% increase in offspring generation, equating to several hundred more offspring available for release to augment natural populations for the species on Mount Baw Baw. Our results highlight the importance of measuring a suite of response variables across different developmental life-stages to properly assess the outcome of hormone therapies. Future research aims to expand the number of fitness-determining traits quantified to include growth trajectories, morphology, and behavioral performance (Silla and Byrne, 2024).

While the proportion of eggs developing to metamorphosis was significantly different between treatment groups, metamorph weight was statistically similar, and individual variation in weight was extremely low. These results are consistent with the notion that Baw Baw frog larvae must reach a body-size threshold in order to trigger metamorphic onset (Gilbert et al., 2020). Previous research in this species found that rearing temperature and food availability

influenced time to metamorphosis, but that there was no effect on body mass or length (Gilbert et al., 2020). The Wilbur–Collins model for amphibian metamorphosis explains that the range in body size and time to metamorphosis for a given species are determined by a minimum body size that must be obtained and a maximum body size that will not be exceeded at metamorphosis (Wilbur and Collins, 1973). The model predicts that amphibian species inhabiting stochastic environments will have a wide range of possible sizes at metamorphosis, while species exploiting relatively stable environments during larval development will have a narrower range. The narrow range in size at metamorphosis for Baw Baw frogs reported in the present study, and by Gilbert et al. (2020), suggests that conditions during larval development on Mount Baw Baw have historically been relatively stable. As natural conditions become increasingly stochastic, Baw Baw frog tadpoles may face new selective pressures and a narrow metamorphic body size range may have negative fitness consequences for remnant wild populations. Consequently, developing effective protocols for larval rearing within the conservation breeding program is likely to become increasingly valuable into the future.

Of note, the percentage of pairs ovipositing reported in the present study (92-100%) was substantially higher than the percentage of pairs ovipositing in the previous breeding season under the same hormone treatments (33-71%) (Silla et al., 2023). The age and body condition of animals was comparable between years, so differences are not expected to be a result of parental phenotype. While the provision of environmental conditions, including breeding enclosure habitat/substrate, food availability, temperature, and photoperiod, remained constant between years, there were two abiotic changes that may have contributed to the differences observed. First male-female pairs in the present study entered the breeding tanks on October 10, 2023, two weeks later than pairs in our previous study, which entered the breeding tanks on September 26, 2022. Frogs in both years were exposed to the same temperature regime and were warmed after an over-wintering period at the same time. The increase in spawning success in the present study may reflect a beneficial effect of a longer warming period to prime the gonads and prepare broodstock for reproduction. The second difference between the two years, was that breeding enclosures were initially irrigated with Reverse Osmosis (RO) water, which was changed to a carbon-filtered water system prior to the present study. Filtration via reverse osmosis is a purification process that removes dissolved solids, including salts, from source water. While species such as the Lake Oku clawed frog (*Xenopus longipes*) are sensitive to dissolved solids and larvae only thrive in RO water (Michaels et al., 2015)), other species develop osmotic imbalance in pure RO and carbon-filtered, or reconstituted RO water is recommended (Odum and Zippel, 2008). Previous research has shown that fertilization success of the terrestrial-breeding anuran *Pseudophryne guentheri* is improved by 20-30% in higher osmolality solutions (25-100mOsm kg^{-1}; generated by serial addition of amphibian saline) compared to pure water (3 mOsm kg^{-1}), reflective of the natural terrestrial fertilization environment of this species (Silla, 2013). The mode of reproduction for the Baw Baw frog similarly involves a terrestrial oviposition site. If female Baw Baw frogs have the ability to assess the osmolality of the environment, as observed in other species (Haramura, 2008), then the provision of RO water may have been suboptimal and resulted in fewer females ovipositing compared to the present study. Overall, the differences in spawning success observed between years highlights the importance of a holistic approach to amphibian captive breeding. While reproductive technologies, specifically hormone therapies, can be used to overcome impediments to breeding that are often observed in captive amphibians, the outcomes of hormone therapies are enhanced when they are used in concert with the provision of naturalistic environmental conditions that have been optimized on a species-specific basis (Silla et al., 2021). The provision of environmental conditions should be viewed as an evolving process as a deeper understanding of species' requirements are elucidated.

5 Conclusions

Our study adds to a growing body of research reporting the effectiveness of utilizing reproductive technologies to improve reproductive outcomes for amphibian conservation management. We have highlighted the need for research that quantifies the impact of hormone therapies on a suite of fitness-determining traits that encompass a greater temporal span in order to gain a more comprehensive understanding of outcomes. Further research in this area will allow us to gain a better understanding of the impact of protocol refinement on individual fitness and the long-term viability of populations, which will ultimately allow us to progress the field of amphibian reproductive technologies to maximize the chance of beneficial conservation outcomes (see Silla and Byrne, 2024). Critically, the present study has taken a step to bridge knowledge gaps concerning how specific hormone therapies may impact offspring fitness by monitoring individuals through to metamorphosis and quantifying size at metamorphosis. Overall, male-female pairs that were administered GnRHa produced a significantly higher total number of eggs and proportion of eggs that developed to metamorphosis, resulting in several hundred more metamorphs available to augment wild populations. We maintain that amphibian CBPs must optimize reproductive output and retention of genetic diversity to ensure the best chance of recovering threatened wild amphibian populations and recommend the continued use of GnRHa to enhance conservation-breeding outcomes.

Data availability statement

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

Ethics statement

The animal studies were approved by Zoos Victoria Animal Ethics Commitee ZV22009. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DeG: Investigation, Project administration, Writing – original draft, Writing – review & editing. DaG: Project administration, Writing – review & editing. PB: Conceptualization, Supervision, Writing – review & editing. AS: Conceptualization, Supervision, Formal analysis, Methodology, 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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Behavioural ethogram to inform *ex-situ* initiatives for a critically endangered bird – the case of the Plains-wanderer

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A thorough understanding of behaviour is essential to a species recovery effort, not only to inform management and husbandry decisions, but critically, to ensure optimum survival of released animals and their offspring. The endangered Plains-wanderer, endemic to Australia and the only extant member of its family, is a bird of great conservation significance. Despite their phylogenetic uniqueness and conservation status, very little is known about their basic behavioural ecology. As part of the National Recovery efforts, an *ex-situ* breeding program was established to create an insurance population with the aim of releasing zoo-bred birds into their natural range. Such programs provide unique opportunities to conduct detailed behavioural and ecological studies. However, such studies are dependent on a comprehensive understanding of basic behaviour and associated social interactions, as well as a common vocabulary across institutions when it comes to describing patterns of behaviour. Therefore, a detailed ethogram is a vital first step. Here we have collated initial behavioural observations and descriptions from three main breeding institutions to create a unified ethogram across sites, with the aim of facilitating future research endeavours. Ultimately a systematic understanding of behaviour will not only improve management and conservation initiatives, but also the understanding of adaptability to potential threats going forward.

KEYWORDS

Plain-wanderer, ethogram, recovery program, conservation outcomes, adaptation, *ex-situ*

Introduction

The functional importance of animal behaviour for the success and effectiveness of conservation management projects has been increasingly recognised in recent years (Greggor et al., 2019, 2020). Conservation breeding and translocation programs have been established to safeguard species from extinction and set up viable breeding populations for future releases, and a vital element to increase the probability of success of these programs is the understanding of the target species' behaviour (Berger-Tal et al., 2019). A thorough understanding of behaviour is essential to a species recovery's efforts (Sutherland, 1998), not only to inform management and husbandry decisions, but critically, to ensure optimal survival of released animals and their offspring. Zoos play a critical role in the recovery of threatened wildlife due to their husbandry, welfare, veterinary and research skillset, and the opportunities they create for in-depth analysis of animal behaviour.

At the early stages of an *ex-situ* breeding program for endangered species, when there's little evidence of husbandry requirements, or a desire to know more about the species' responses to husbandry and management (Rose and Riley, 2021), the understanding of species-specific behaviours becomes critical. An ethogram is a basic tool to the understanding of animal behaviour by clearly defining, describing, and classifying distinct behaviours commonly exhibited by a species (Fern et al., 2022). To ensure successful breeding and reintroduction programs it is important to establish universal, standardised ethograms that can be used by multiple programs and institutions to compare behaviours of both wild and captive populations and understand breeding behaviour to increase the likelihood of success. When all partner institutions refer to the same behaviours, it increases objectivity and allows for comparisons among them. Recent calls for consistency in defining and coding animal behaviour have stemmed from variation in how behaviours are defined within and across species, which could have implications for reproducibility and comparability of results (Fern et al., 2022).

The Plains-wanderer, *Pedionomus torquatus*, is a ground-dwelling, native grassland specialist bird endemic to Australia (Baker-Gabb et al., 2016), and the sole member of the Pedionomidae family. The species is endangered and considered of high conservation significance (BirdLife International, 2022; Jetz et al., 2014; McClure et al., 2023). Population declines in the wild have mostly been associated with the conversion of native grasslands to crops and dense introduced pastures (Commonwealth of Australia, 2016) and climate extremes, both drought and excessive wet years (Antos and Schultz, 2019; Parker et al., 2021). Current population estimates across its range suggest a wild population of less than 500 individuals and trending downward (Antos and Schultz, 2020). Plains-wanderers are solitary and occupy home ranges of about 12ha (Baker-Gabb et al., 1990); uncommon among birds, females are larger and more conspicuous than the males. Plains-wanderers are considered polyandrous, and the male takes the major role in incubating and caring for the young (Bennett, 1983). Plains-wanderer tends to forage in denser grassland patches than those preferred as nocturnal roosting sites (Nugent et al., 2022). Due to the elusive nature of the Plains-wanderer and resulting challenges with direct observations, little is known about its behavioural repertoire (Bennett, 1983; Baker-Gabb, 1988; Nugent et al., 2022), limited to published notes of chance encounters (Purnell, 1915; Hopton and Carpenter, 2021) or as unpublished anecdotal records.

As part of the recovery efforts for the Plains-wanderer (Commonwealth of Australia, 2016), an *ex-situ* conservation breeding program was established to safeguard the species from extinction and set up a viable breeding population for future translocations to its original range. While information can be gathered from wild populations, for this cryptic species, the *ex-situ* population provides a unique opportunity to observe, document and better understand its breeding behaviour, including different aspects of male-female interactions, courtship behaviour, and parental care of wild birds which can be used to 'benchmark' wild-type behaviours to assess potential changes over time attributable to a captive environment and inform adaptive management of the program.

We combine behaviour observations from video footage from three different zoo-based breeding population across NSW and Victoria and establish a basic ethogram from Plains-wanderer collected from the wild (potential founders). It is hoped this information will support the species' *ex-situ* management to ensure that individuals of the insurance population maintain natural behaviours and are as suitable as possible for release. We further aim to discuss the application of ethograms in the management of *ex-situ* breeding program and provide a framework that can be applied in other recovery programs.

Material and methods

Wild adult Plains wanderers (n=25) were brought into three separate breeding facilities (Taronga Zoo, Sydney; Taronga Western Plains Zoo, Dubbo; and Werribee Open Range Zoo, Werribee) between June 2017-2020. Wild birds were located and captured during nocturnal spotlight surveys from a slow moving (<5 km/hr) vehicle. While Plains-wanderers are typically easy to approach, not flighty and able to be captured by hand, a lightweight insect net (with a padded rim and small mesh) was used to capture the birds, which were transported to the breeding facilities in customised transport boxes. The behavioural observations were initiated soon after each bird was brought into the holding facilities to capture as much wild representative behaviour as possible. Due to their cryptic behaviour, Plains-wanderer are difficult to observe directly therefore, to allow for husbandry monitoring of the birds, all aviaries were fitted with CCTV cameras operating for 24 hours (about 50% of the cameras had night vision capabilities). The behaviours were analysed by remote monitoring and review of the CCTV cameras footage.

During the observation period (2017-2020) birds were housed individually or in pairs in designated aviaries that varied in size from 2.8m x 3.2m x 2.4m to 4.0m x 4.0m x 4.0m, vegetated with plant species typical of Plains-wanderer habitat to emulate conditions in the wild. This configuration could change for management purposes (e.g. birds held in small single-sex groups (3-4 birds) in each aviary).

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Observations

Initial behavioural observations were obtained from CCTV cameras recording for 24 hours, 7 days a week in each aviary across the three sites within the first 12 months of the wild birds arriving at the breeding population. Birds were out of sight or hidden from view for large portions of the recordings, therefore behaviours were described from *ad lib* samples when a bird was visible as opposed to fixed-time scan sampling.

During the description of behaviours and development of the ethogram, the footage and descriptions of behaviours were exchanged between the institutions to validate their meaning and seek common description (as a proxy for interobserver reliability).

Results

The ethogram presented here includes four breeding seasons' worth of displays from both male and female birds, as well as calling and social interactions, and general maintenance behaviour. Overall, more than 10,000 hours of footage was reviewed across the three institutions to establish the ethogram.

From the initial data, results indicate that most mating and reproductive-related behaviours occur during the twilight hours, with many displays between birds occurring between early morning (dawn) and evening (dusk). Although only a subset of the behaviour has been analysed due to the extensive surveillance system, and the cryptic nature of the species, pin-pointing this time of day for analysis going forward is already a valuable outcome.

Forty-four different behaviours were described in the ethogram compiled by Pauligk (2020), with an additional category of 'other behaviours' describing less frequently observed behaviours.

Here we grouped some of these behaviours into five broad functional categories (Sexual, Communication, Anti-predator, Maintenance, Movement) and discuss some of the most typical ones (Table 1). Videos of specific behaviours can be found in the Supplementary Material.

Discussion

Here we establish an ethogram of 'wild-type' behaviour that has been observed in wild-born Plains-wanderers brought into the *ex-situ* breeding program. The behaviours described here are by no means exhaustive, however due to their relative regularity between individuals and across institutions, we suggest these behaviours provide the main repertoire as seen in wild Plains-wanderer. In some cases where function is currently speculative, we have tried to include form as a means of avoiding possible confusion when assessing behaviour going forward. The development of the ethogram allowed for consistency and standardisation of the behaviours' description and meaning to ensure objectivity and uniformity of terminology. Thus, they can be used as a benchmark of wild behaviours against which the behaviour of birds within the *ex-situ* population can be compared.

TABLE 1 Ethogram developed for the critically endangered Plains-wanderer based on CCTV-captured footage at three breeding institutions (adapted from Pauligk, 2020) as part of the recovery program.

Category	Behaviour	Description
Sexual		
Courtship	Circling*	Male and female closely face each other and move around each other in tight circles, clockwise and anti-clockwise. Typically, precedes an attempt to mount by the male. Usually, the chest is lower than the tail, however instances of chest bumping (see below) may be apparent also.
	Aeroplane*	Female lowers chest to the ground with head extended forward. Wings hung outwards from body and lateral primary feathers drag along the ground. Posture gives the female appearance of looking larger than she is.
	Cloaca display	Female exhibits her cloaca to the male.
	Bowing	Female stands tall on her toes and pulls her body in tight. She then lowers her head down towards the ground in front of her, sometimes with a little shake of the head and eyes closed.
Breeding	Mounting*	Male mounts female from behind and typically grips her neck feathers with his beak. Female usually lowers herself to the ground and lifts her tail.
	Incubating	Sitting on the eggs and rolling them several times a day. Short breaks are taken during the day to feed; no breaks are taken during the night. Mostly displayed by the male, however some females also play a small role in incubation.
	Brooding/ chick rearing	After the chicks hatch, the male let them sit under his body. The male calls the chicks over, where two will refuge right under his body side by side, and the remaining two will tuck in, one under each wing.
Communication		
	Chest bumping*	Either of the pair puffs out their chest and either passively waits for the other to bump into them, or actively walks towards the other whilst in close proximity. Typically, both individuals will puff up their chest simultaneously.
	'Ooom' vocalisation*	Female vocalisation in which she flattens her body in a diagonal position with head extended. Neck area inflates and deflates, while a deep "ooom" sound is emitted with her mouth closed.
	Wailing vocalisation	Male vocalisation in which he flattens his body in a diagonal position and extends his head. Neck inflates and deflates, while a high pitched 'haunted wailing' sound is emitted from his nostrils.
	Side jumps	Typically accompanied by a vocalisation, this behaviour is performed by both sexes. It involves a single flap of the wings and short lift to jump from side to side.

(Continued)

TABLE 1 Continued

Category	Behaviour	Description
Anti-predator		
	Elevated viewing	Individuals moving up into grasses or herbs, off the ground.
	Fright response (hunker down)	Dropping suddenly to the ground, flattening body to ground, and remaining still. Can last from one second to over one minute.
	Flush	Individual suddenly flies straight up into the air, from the ground upwards. Can be controlled or uncontrolled.
Maintenance		
	Foraging	Actively consuming food items from within the aviary, as opposed to from food bowls.
	Drinking	Consuming droplets from vegetation or pecking at water bowl or trough.
Movement		
	Walking	Slow movement with short quick darting movements, often in a zigzag motion with head bobbing, tall posture.
	Wing walking	Bird walks with wings extended in an arched triangular position. Wings can be stationary or shaking. Short, stuttered movements, usually at night.
	Jump fly	Distinct from flush, no perceived fright stimulus
	Flutter	In an open position, the bird lifts its body between one and 30cm off the ground with rapid wing flapping, returning to the ground repeatedly.

*-Videos uploaded as Supplementary Material.

There is no detailed description of Plains-wanderer behaviours in the wild which prevents comparisons with the behaviours exhibited by the birds in the *ex-situ* population. However, as the behaviours described in the ethogram were based on observations of wild birds collected for the insurance population and recorded soon after their transition into the *ex-situ* program, we assume they are representative of the behavioural suite exhibited by Plains-wanderer in natural habitats. For example, observations of mating behaviours during the twilight hours mirror observations made during field-based activities (David Parker, pers.obs.). Further, Plains-wanderer commonly consumed water droplets on leaves after rain or irrigation, not just from water bowls provided, which suggests this is how they access water in the wild.

The establishment of this ethogram is especially relevant as the Plains-wanderer recovery program is reaching the next stage of trialling wild translocations to boost natural populations. Considering that the phenotypic quality of captive-bred animals is critical in determining the success of reintroduction programs (Tripovich et al., 2021; Crates et al., 2022), the more we understand and agree on typical behaviours the more informed/evidence-based decisions will be about release cohorts or release candidate suitability.

Identifying the behavioural factors that determine current and future reproductive success is the obvious next step when analysing Plains-wanderer behaviour. Currently data is being collated from all successful mating events across all institutions to retrospectively determine which factors may have contributed to successful mating. Once these factors are established, we can investigate the role that behavioural matching and mate-choice has on factors such as clutch size and chick health. Recent evidence has shown that incorporating behavioural compatibility and mate-choice into breeding programs enhances reproductive output and survivorship (Hartnett et al., 2018; Martin-Wintle et al., 2018).

Further, it is known that captivity can cause changes in important life history and behavioural traits in various species over time (Crates et al., 2022), thus putting individuals earmarked for release potentially at a disadvantage upon reintroduction to the wild. A full grasp on typical behaviours will inform management decisions regarding the most suitable candidates for release as well as the release protocol. For example, the Regent Honeyeater (*Anthochaera phrygia*), another critically endangered Australian bird currently being managed through a conservation breeding program, has been bred in zoos for approximately 20 years and over 400 individuals released to the wild. Over that time changes have been observed in the vocalisations of the species both in captivity and the wild, likely due to both small populations limiting learning opportunities and mimicry of other, more abundant species (Crates et al., 2022). A recent study (Appleby et al., 2023) indicated that female Regent Honeyeaters tend to prefer songs of males they are familiar with. Further, analysis of the fitness of released individuals highlighted the importance of song tutoring, as well as complex pre-release zoo habitats (Tripovich et al., 2021). Behavioural management has now been implemented to monitor and enhance the fitness of birds and assist in selecting release candidates.

The detailed behavioural observations and resulting development of the Plains-wanderer ethogram provide a consistent framework for using typical behaviours as benchmarks to inform release candidate selection. For example, by comparing behaviours observed as part of the ongoing husbandry (such as foraging) to behaviours when pre-release changes are introduced (such as consistent foraging behaviour when scatter feed is increased or changes in behaviour following attachment of a tracking device), then birds that are behaviourally suitable for release, in addition to being demographically and genetically suitable, can be confirmed. These traits may include the inability to recognise natural foods or predators, or deficient locomotor skills and/or spatial awareness, which, if recognised in advance, may provide an opportunity to address potential deficiencies in a release cohort by, for example, introducing pre-release training. Whether this is the case for bird species is largely unknown and thus this project provides a unique opportunity to intensively monitor the behaviour of birds from the time they enter the *ex-situ* population, to the time their descendants can be released.

As *ex-situ* breeding programs increasingly become an essential component of the recovery efforts for 'at-risk' wildlife, all information on typical aspects of the species biology is essential, especially for evolutionary unique species for which relying on 'proxy' species is difficult, as is the case for the Plains-wanderer. The systematic description of behaviours in ethograms brings rigour and consistency to *ex-situ* breeding programs by removing inherent subjectivity and variation due to different stakeholders. Further, the implementation of

systematic behaviour observations and descriptions allow for monitoring if and how behaviour changes over time. This would allow for adaptive management changes within the breeding program to avoid behavioural changes within a captive environment and ensure birds for release are as behaviourally sound as possible.

Data availability statement

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

Ethics statement

The animal study was approved by Taronga Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MVS: Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing. YP: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. AB: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. MO'R: Data curation, Investigation, Validation, Writing – review & editing. RM: Investigation, Validation, Writing – review & editing. CH: Investigation, Supervision, Validation, Writing – review & editing. BP: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, 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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Supplementary material

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Fecal microbiota is more stable during degradation and more diverse for *ex situ* cheetahs in Namibia compared to the USA

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The relationships between gut microbiota and animal health are an important consideration increasingly influential in the management of wild and *ex situ* endangered species, such as the cheetah (*Acinonyx jubatus*). To better understand these relationships, fresh fecal samples are currently required as a non-invasive alternative for the gut microbiome. Unfortunately, fresh samples are challenging to collect in the wild. This study had two aims: 1) to determine the optimal collection time point for cheetah feces after deposit in their native environment of Namibia as a guide for wild cheetah fecal microbiome studies; and 2) to compare the fecal microbiota of two *ex situ* cheetah populations (Front Royal, VA, USA and Otjiwarongo, Namibia), which also consume different diets. We collected eight fresh fecal samples from cheetahs in Namibia and allowed them to decompose for four days, taking subsamples each day. The fresh Namibian samples (n = 8) were also used in objective two for comparison to fresh USA cheetah samples (n = 8). All samples were analyzed for bacterial community diversity and composition using 16S rRNA gene amplicon sequencing. First, over a five-day sampling period in Namibia, subsamples 1-3 days post-fresh showed no changes in bacterial diversity or composition compared to fresh subsamples. Second, fresh *ex situ* cheetah samples under Namibian conditions had increased bacterial taxa, more phylogenetically diverse bacterial communities, and compositionally distinct microbiomes from cheetahs managed in human care in the USA. However, when bacterial ASVs were weighted by relative abundance, both populations shared 69% of their total bacterial sequences indicating a conserved cheetah microbiota between the two populations. We also found few differences in predictive functions of the fecal microbiota between the populations, where only one disease-related pathway was higher in the USA samples. Overall, our findings suggest that in dry season conditions (no recorded rainfall) in Namibia, fecals may be usable for up to three

days after defecation for microbial ecology studies. There are significant differences between *ex situ* Namibian and USA populations, and we suggest further investigation into the influence of diet, host demographics, and environment on the gut microbiota and health of cheetahs.

KEYWORDS

***ex situ* carnivores, cheetah, non-invasive sampling, microbial stability, gut microbiome, Namibia**

1 Introduction

Microbiome studies are becoming an essential part of conservation biology. There is a plethora of evidence supporting the role of gut microbiomes in wildlife health and survival (Bragg et al., 2020; Cabana et al., 2019; Sugden et al., 2020; Gillman et al., 2022; Redford et al., 2012; Clayton et al., 2018; Bragg et al., 2020). Many microbiome studies on non-domestic animals focus on *ex situ* individuals because it is easier to control experimental variables and ensure timely and accurate sample collection. However, to obtain a better understanding of the complex dynamics between hosts and their gut microbiomes it is also important to study animals in their natural habitat (Amato, 2013). The current gold standard for non-invasive gut microbiome studies is to collect fresh fecal samples to characterize fecal microbiota as a stand-in for gut microbiota. Unfortunately, collecting fresh feces in the wild is quite difficult for many elusive, dangerous, or far-ranging species, including the cheetah (*Acinonyx jubatus*).

Due to the difficulty in collecting fresh fecal samples, more studies are investigating the temporal stability of fecal microbiota post-defecation. These studies aim to characterize shifts in fecal microbiota once they are exposed to the environment and to identify the timepoint after which the fecal microbiota no longer represent that of a fresh sample. Many studies in various species found evidence for shifts in microbial diversity or composition within five or less days of excretion (Lafferty et al., 2022; Menke et al., 2015; Beckers et al., 2017; Wong et al., 2016), including our previous study on cheetahs in the USA that demonstrated changes after one day post excretion when in moist conditions (Maly et al., 2024). Other studies report no changes at all over the four days of their experiment (e.g., Tal et al., 2017). Because of the large variation in reported timelines of fecal microbial changes, there is no general rule for how fresh is 'fresh enough' in fecal microbiome studies. It is recommended that temporal experiments be performed for each new species (Menke et al., 2015), but we suggest that environmental conditions should also be considered (Maly et al., 2024). We therefore stipulate that our previous study on fecal microbiome stability may not be representative for the entire species as it was performed on *ex situ* cheetahs in a US facility, which may differ from the fecal microbial stability of cheetahs in their native environment. Before we can compare fecal microbiomes of wild and *ex situ* cheetahs through non-invasive collection, we need a better understanding of how long fecal samples remain stable in the arid and hot climate of Namibia, within the natural range of wild cheetahs.

While microbiome studies in the wild provide important clues for conservation, microbiomes are also relevant for *ex situ* wildlife. Recently, there has been concern for potential dysbiosis of managed animal microbiomes as gut flora can be shaped by management practices through biotic and abiotic factors such as diet (Bragg et al., 2020; Haworth et al., 2019; Gibson et al., 2019), administration of antibiotics and other veterinary care (He et al., 2018; Dahlhausen et al., 2018), exposure to humans and the built environment (Cheng et al., 2015; Hyde et al., 2016; Wan et al., 2016; West et al., 2019), reduced exposure to conspecifics (Tung et al., 2015), and higher density of animals that may not naturally be in proximity to each other (de Jonge et al., 2022; McKenzie et al., 2017). A high incidence of gastrointestinal (GI) diseases in managed cheetahs contributed to the majority (40-60%) of deaths and euthanasia in captivity between the 1980s and 2000s (Munson, 1993; Munson et al., 2005, 1999; Terio et al., 2018). Given its involvement in GI health and inflammatory responses in many other species, there is a growing interest in characterizing the gut microbiome of *ex situ* cheetahs in relation to these health issues. Lower microbial species diversity and increased temporal microbial variation have been reported in *ex situ* cheetahs suffering from GI distress, compared to those that are healthy (Becker et al., 2015). These findings are similar to reports comparing healthy and GI-inflamed domestic cats (Honneffer et al., 2014; Janeczko et al., 2008). Previous studies in cheetah gut microbiomes suggest there may be population differences across wild Namibian (Wasimuddin et al., 2017; Menke et al., 2014), European *ex situ* (Becker et al., 2014), and USA *ex situ* (Maly et al., 2024) cheetahs. These existing studies span a variety of collection, processing, and analytical methodologies, making it difficult to make direct comparisons.

In this study, our first objective was to determine how many days after defecation the fecal microbiota remained representative of a fresh fecal in managed cheetahs living in Namibia as a proxy for wild cheetah fecal microbiome sampling. Due to difficulty in collecting fresh fecal samples from the wild, collecting samples from cheetahs in a managed facility within their native home range offers a next-best opportunity. Cheetahs at the Cheetah

Conservation Fund (CCF) in Namibia live in large outdoor-only enclosures and are exposed to the same environmental elements as those in the wild. Our second objective was to investigate the differences in fresh fecal microbiota between cheetahs in managed facilities in the USA compared to those in Namibia to understand the effects of environment and diet on the cheetah gut microbiome. We therefore utilized CCF and USA populations to learn more about environment and diet effects on the fecal microbiota.

2 Methods and materials

2.1 Sample collection

2.1.1 Fecal microbial stability

We collected eight fresh fecal samples from wild-born cheetahs living at the CCF center in Otjiwarongo, Namibia. Cheetahs lived in outdoor-only enclosures with native trees and plants, as well as shade structures for cover. They were fed a raw diet of donkey and horse meat on the bone, with skin and small bones removed, and vitamin and mineral supplement powder rubbed onto the meat (Predator Powder, V-tech, Midrand, South Africa). Their feeding regime included one fasting day a week. Fresh water was available *ad libitum*. Cheetah ages ranged from 3-14.5 years and included both males and females. Fresh fecals were collected as observed over the course of four days between 9 am and 5 pm. All defecation events were witnessed and fecals removed from the enclosure within 30 minutes. Samples were placed in an adjacent area used as the experiment plot to allow ease of access for continued subsampling. We positioned a generic mesh window screen (Supplementary Figure S1) over the top of the feces to prevent dung beetles, and other animals from taking the remaining feces, but allowing other natural environmental exposure processes to occur. Each day, we removed a one-inch subsection from the end of the fresh fecal with a sterile scalpel and further subsampled the interior core of the one-inch piece with sterile forceps and a second sterile scalpel. The remainder of the feces was placed back in the experimental plot. The first subsample was considered Day 0 (fresh). The subsampling procedure was repeated every 24 h, for four consecutive days (Day 1 – 4) or until the feces ran out (Table 1). We recorded the maximum daily rainfall (cm), humidity (%), and temperatures (C) for each day of collection (Supplementary Table S1). Subsamples were placed in a -20°C freezer until processing.

2.1.2 Comparison of fresh samples from two populations

To compare the microbial ecology of two cheetah populations we used only the fresh subsamples from the above Namibian (NAM) collections (n = 8). In addition, we used eight fresh subsamples from a similar and previously published study collected from the Smithsonian's National Zoo and Conservation Biology Institute (Front Royal, VA, USA) as described (Maly et al., 2024). Samples from the US-based study were renamed from the Aju (for *Acinonyx jubatus*) moniker (Aju1, Aju2, etc.) to USA (USA1, USA2, etc.) as in Maly et al. (2024). USA cheetahs lived in outdoor enclosures with access to indoor spaces. The diet for cheetahs at the USA institution was primarily a commercially available ground beef diet that includes beet pulp (Nebraska Premium Canine Diet, North Platte, NE, USA) with the addition of weekly whole rabbits (with fur) and horse bones that contain small amounts of meat and cartilage (~ 200g) but no skin or fur. Of the eight USA fecals collected, two were from adult (6 years old) females while the remaining six samples came from male and female pre-pubertal juveniles (~1 year old), though sex was not noted for these six individual samples.

2.2 Sample DNA extraction and library prep

DNA was extracted at the Namibia-based CCF Conservation Genetics Laboratory from 0.25 g frozen feces using the QIAamp PowerFecal DNA Kit (Qiagen, MD) following manufacturer's instructions. For each batch of sample extractions, a negative control was included and carried alongside the other samples throughout the experiment, to identify potential extraction contaminants. Following extractions, DNA concentrations were measured with a nanophotometer (Implen NP80-Touch; Implen, Munich, Germany).

Fecal bacterial DNA was amplified following a previously published two-step polymerase chain reaction (PCR) protocol with dual-index paired-end Illumina sequencing (Keady et al., 2021). The PCR amplified the V4-V5 region of the 16S rRNA gene using universal primers 515F-Y (GTGYCAGCMGCCGCGGTAA) and 939R (CTTGTGCGGGCCCCCGTCAATTC) (Muletz Wolz et al., 2018). PCRs were performed in duplicate for each sample, including the negative extraction and PCR controls. To lower the risk of contamination during transit from Namibia to the USA, the PCR was performed at CCF and PCR products shipped on ice to the Center for Conservation Genomics (CCG), Smithsonian National Zoo and Conservation Biology Institute. Duplicate PCR products were combined before being purified with magnetic beads. After the first bead purification, samples were indexed with i5s and i7s, cleaned again, quantified, and pooled as specified in Keady et al. (2021). An agarose gel was run of the 16S rRNA library and the target band (~578 base pairs) was isolated and removed using a QIAquick Gel Extraction Kit (<ns/>28704, Qiagen, MD) and diluted to 4 nM. All samples were sequenced using an Illumina MiSeq (v3 chemistry: 2 x 300 bp kit) at CCG. For our second objective, to avoid any bias from sequencing, all Day 0 (fresh) sample libraries from USA and NAM collections were pooled and re-sequenced together on an Illumina MiSeq (v3 chemistry: 2 x 300 bp kit) at CCG.

2.3 Sequence data processing

2.3.1 Fecal microbial stability

Demultiplexed Illumina Miseq sequencing reads were imported into R version 4.0.3 (Team, 2022) using RStudio (v 2022.12.0 + 353). We utilized R package "*dada2*" version 1.16.0

TABLE 1 Sample collection summary for each fecal sample series.

Fecal Sample ID	Day 0	Day 1	Day 2	Day 3	Day 4
NAM 1	122,021 (reads)	116,183	128,746	139,582	140,909
	142 (SR)	155	154	138	144
	6.32 (PD)	7.07	6.37	6.27	6.39
NAM 2	71,275	129,347	175,646	98,324	222,683
	263	192	292	151	190
	11.67	9.63	11.92	8.09	9.18
NAM 3	93,988	105,431	110,887	156,251	176,384
	152	158	161	180	152
	6.4	6.8	6.35	7.05	6.83
NAM 4	144,748	150,493	151,050	242,850	sample ran out
	126	181	146	187	NA
	5.91	7.95	6.58	8.5	NA
NAM 5	120,115	118,943	220,673	304,489	**8,390**
	161	174	178	194	NA
	7.45	7.09	8.83	7.81	NA
NAM 6*	166,875	**5,479**	173,683	135,001	**5,076**
	138	NA	128	157	NA
	7.07	NA	6.74	7.10	NA
NAM 7	204,277	154,511	108,879	131,564	**47**
	164	159	144	164	NA
	6.14	6.19	6.23	6.9	NA
NAM 8	176,021	140,537	126,256	219,849	330,652
	129	129	137	166	168
	5.83	6.48	6	6.77	6.28

For each subsample the total post filtered read counts (reads), species richness (SR), and Faith's phylogenetic diversity (PD) are listed. Read counts in bold indicate sequence counts before final filtering, these sample were removed during the filtering step (removed samples < 70,000 reads). Asterisk (*) indicates individual was dropped from first study objective due to non-sequential samples.

(Callahan et al., 2017, 2016) to merge paired ends, remove chimeras, and filter out low quality reads (maxEE > 2). Filtered and merged sequences from two sequencing runs were combined to generate amplicon sequence variants (ASVs) and assign taxonomy using the Ribosomal Database Project [RDP (Wang et al., 2007)] 16S training set (set 16, release 11.5). A phylogenetic tree was built using Quantitative Insights Into Microbial Ecology 2 [vQIIME2-2020.8; (Bolyen et al., 2019)] using FastTree (Price et al., 2009). We imported the ASVs, taxonomy table, phylogenetic tree, and metadata into a phyloseq object (McMurdie and Holmes, 2013) for processing. We removed putative contaminant sequences using the combined Fisher method with a threshold of 0.1 in the R package "*decontam*" [v1.18.0 (Davis et al., 2018)]. We removed ten contaminant sequences and then filtered out singleton ASVs (ASVs that occur in only one sample), ASVs classified as Cyanobacteria, negative control samples, and low sequence count (< 71,275 reads) samples. After quality control and filtering, the sequencing depth variation (max/min) was 4.6 fold (max = 330,423, min =71,066).

2.3.2 Comparison of fresh samples from two populations

For population comparisons, sequence reads from the fresh Namibian (NAM) and USA (USA) samples were combined into a phyloseq object after filtering and merging and were assigned taxonomy together using QIIME2 [vQIIME2-2020.8; (Bolyen et al., 2019)] using FastTree (Price et al., 2009). After taxonomy assignment, we split the phyloseq object in two based on location (NAM and USA) for *decontam* to remove contaminant sequences separately. While the two populations were indexed, prepped for sequencing, and sequenced in the same place (CCG) and on the same sequencing run, the samples were collected and extracted in different locations (CCF in Namibia and SCBI in USA) and may have different background contamination. The number of contaminants removed were three and one from the fresh NAM and USA samples, respectively. After *decontam*, data were merged back together into a final clean phyloseq object. After cleaning, we compared sequencing depth across samples. We found sequencing

depth variation (max/min) was 26.3-fold between the highest sample and lowest sample (max =83,479, min =3,175). Based on current literature (Weiss et al., 2017), we rarefied all samples to the lowest sequencing depth (3,175).

2.4 Statistical analyses

Statistical analyses were performed in RStudio for R. Significance for all analyses was set to $p < 0.05$ and we adjusted p-values for multiple comparisons using Bonferroni. Analysis pipelines for characterizing microbial structure and composition were based on previous research (Muletz-Wolz et al., 2019a, 2019b; Keady et al., 2021; Bragg et al., 2020). For both, comparisons across sampling days and between two populations, we conducted two metrics of microbial diversity which included alpha diversity (within sample variation), beta diversity (between sample variation), and the changes in relative abundance at the ASV and Phyla levels. For comparisons of fresh samples by population we also predicted functional pathways based on marker gene sequences using PICRUSt2 and linear discriminant analyses effect size using LefSe.

2.4.1 Fecal microbial stability

2.4.1.1 Relative abundance, microbial diversity, and composition

Fecal ID NAM 6 was omitted from this objective, due to an incomplete series collection (Table 1). Relative abundance was measured using the package *phyloseq* (McMurdie and Holmes, 2013) using the function tax_glom() at the phyla level and merge_samples() function by Sample Day. Differential abundance among sample days was calculated with raw ASV counts using Multivariable Association in Population-scale Meta-omics Studies (MaAsLin2) software (Mallick et al., 2020, 2021). *MaAsLin2* was performed on the ASV, family, and phylum levels. For the family and phylum levels, the full dataset was collapsed to the appropriate levels using the tax_glom() function in *phyloseq*. For all levels, the model included Sample Day as a fixed effect (reference category = Day 0) and Fecal.ID as a random effect, where max significance for Benjamini-Hochberg adjusted p values (q values) was set to > 0.05 and all other parameters were set to default.

We examined changes in microbial diversity over time (sample days) using two alpha diversity metrics, species richness (SR) and Faith's phylogenetic diversity (PD). SR is the number of unique ASVs in a sample and PD measures the amount of biodiversity based on the phylogenetic relationships of the taxa and the total tree branch length of ASVs in a sample (Faith, 1992, 2018). Faith's PD was calculated for each subsample with the R package "*picante*" (Kembel et al., 2010). Using the "*lme4*" R package (Bates et al., 2015), we performed mixed effects linear models, with SR or PD as the response variable, sample day as a fixed effect, and fecal ID as the random effect. SR and PD distributions met assumptions of normality (Shapiro-Wilk) and homoscedasticity (Levene).

To identify differences in community composition between subsamples across sampling days we measured Bray-Curtis (abundance weighted taxa), Jaccard (presence-absence of taxa), and unweighted UniFrac (presence-absence with inclusion of phylogenetic relationships of taxa) distances. We used PERMANOVAs (Anderson, 2017) in the package "*pairwiseAdonis*" (Martinez Arbizu, 2020) using the adonis2() function where Bray-Curtis, Jaccard, and unweighted Unifrac distances were the response variable, sample day was the explanatory variable and fecal ID was the random effect. *Post hoc* analyses were performed in the same package using the pairwise.adonis2() function and *p*-values adjusted using Bonferroni. To identify whether dispersion of microbiota composition differed among sample days we used PERMDISP from the package "*vegan*", betadisper() function (Oksanen et al., 2022).

2.4.2 Comparisons of fresh samples from two populations

2.4.2.1 Relative abundance, microbial diversity, and composition

We measured SR and Faith's PD between the two populations as defined above. Using the "*lme4*" R package (Bates et al., 2015), we performed linear models, with SR or PD as the response variable and Population (NAM, USA) as a fixed effect. SR and PD distributions met assumptions of normality and homoscedasticity (Levene). For beta diversity, we again used the same metrics as described above to identify microbial compositional differences between the two populations. We used PERMANOVAs (Anderson, 2017) in the package "*pairwiseAdonis*" where Bray-Curtis, Jaccard, and unweighted Unifrac distances were the response variable and population (NAM, USA) was the explanatory variable. To identify whether dispersion of microbiota composition differed between the populations we used PERMDISP from the package "*vegan*", betadisper() function. Relative abundance was measured using the package *phyloseq* (McMurdie and Holmes, 2013) using the function tax_glom() at the phyla level and merge_samples() function by Population. We used ps_venn() function in the "*MicEco*" package (Russel, 2021) to identify unique and overlapping ASVs by population via raw counts and ASVs weighted by abundance (where ASVs with greater abundance carry a larger influence, weight = TRUE).

2.4.2.2 Enriched microbial taxa analysis

We used Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (Segata et al., 2011) in the R package "*microbiomeMarker*" (Cao et al., 2022) to identify microbial taxonomies enriched in one of the two populations. The threshold minimum LDA score was set to 4 to filter out features with lower effect sizes and detect microbial features with potentially more biologically meaningful significant differences between the two cheetah populations.

2.4.2.3 Predictive functional analysis using PICRUSt2

To identify potential functional relevance of the microbial community differences between the two cheetah populations we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2, Version 2.5.1; Douglas et al., 2020).

We used the Kyoto Encyclopedia of Genes and Genomics (KEGG) database to assign predicted functions and metabolic networks of the bacterial communities (Caspi et al., 2016). Statistical analyses were performed in the Statistical Analysis of Taxonomic and Functional Profiles (STAMP v2.1.1) software (Parks et al., 2014) to identify significant functional KEGG groups at three classification levels between the two populations. We performed Wilcoxon tests to compare functional groups between the populations and adjusted p-values using the Benjamini-Hochberg false discovery rate (FDR) method to account for multiple hypothesis testing. Results were reported as the mean ± standard deviation of the proportion of sequences assigned to the category by population (USA or NAM). Significance was set to $p < 0.05$.

3 Results

3.1 Fecal microbial stability

We obtained 5,031,818 high-quality sequences from 32 samples (Table 1). Individual NAM 6 was dropped from the degradation study because of low sequence yields in subsequent samples which led to non-consecutive sampling. The average number of sequences per sample was 157,244 (range 71,066 – 330,423). There were 576 ASVs from eight phyla including Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria and to a lesser extent (≤ 2 ASVs) Candidatus Saccharibacteria, Chloroflexi, and Deinococcus Thermus (Figure 1A). The seven fresh subsamples (Day 0) were used as the best representatives of the cheetah gut microbiota in our study, and they consisted of 193 ASVs from five Phyla including Firmicutes (41.2% pooled abundance, range across samples 28.4 - 69%, 114 ASVs), Bacteroidetes (28.1%, 3.6 - 46.9%, 34 ASVs), Fusobacteria (16.4%, 7 – 27.7%, 14 ASVs), Actinobacteria (11.4% 4.8-23%, 18 ASVs), and Proteobacteria (2.9%, 0.65 – 7.5%, 13 ASVs). Only the Phylum Actinobacteria was differentially abundant across days, with lower abundance in Day 4 compared to Day 0 (effect size = -1.64, standard error= 0.43, p.adj = 0.029). Within the Phylum Actinobacteria, no families or abundant ASVs were differentially abundant, suggesting that temporal decay is impacting only the higher-level taxonomic distribution and not one particular family within this phylum. Further, across all bacteria and sampling days, we did not detect any differentially abundant taxa at either the ASV or family level.

Bacterial communities remained similar over time, with little variation between fresh fecal samples at Day 0 and subsequent days. Within samples (alpha diversity), both SR (Supplementary Figure S2A) and Faith's PD (Figure 1B) remained similar across the fresh and the subsequent sample days (Table 1). Bacterial SR was 162.43 ± 46.71 (mean ± standard deviation) and PD was 7.10 ± 2.08 at Day 0 with minimal changes thereafter (GLMMs, SR: $x^2 = 0.7639$, $df = 4$, $p = 0.943$; PD: $x^2 = 0.958$, $df = 4$, $p = 0.916$). Similarly, microbial composition did not change between samples over time in presence absence measures (PERMANOVA Sample Day: Jaccard Pseudo-$F_{4,27} = 0.5638$, $R^2 = 0.07709$, $p = 0.064$, UniFrac Pseudo-$F_{4,27} = 0.4034$, $R^2 = 0.05639$, $p = 0.194$; Supplementary Figures S2B, C, respectively). Microbial composition did however vary over time for abundance weighted composition (PERMANOVA Sample Day: Bray-Curtis Pseudo-$F_{4,27} = 0.9866$, $R^2 = 0.12752$, $p = 0.009$; Figure 1C); although in pairwise *post hoc* analyses, no days were significantly different from each other including all later sample days (Days 1-4) compared to Day 0 (*post hoc* Bray-Curtis $p\ adj > 0.156$). This indicates no two sample day comparisons show large enough differences to be considered significant and the global effect of the PERMANOVA may be driven by subtle differences undetected in the pairwise comparisons. Additionally, pairwise testing by nature uses smaller sample sizes which may limit power. Within group dispersion of community composition was

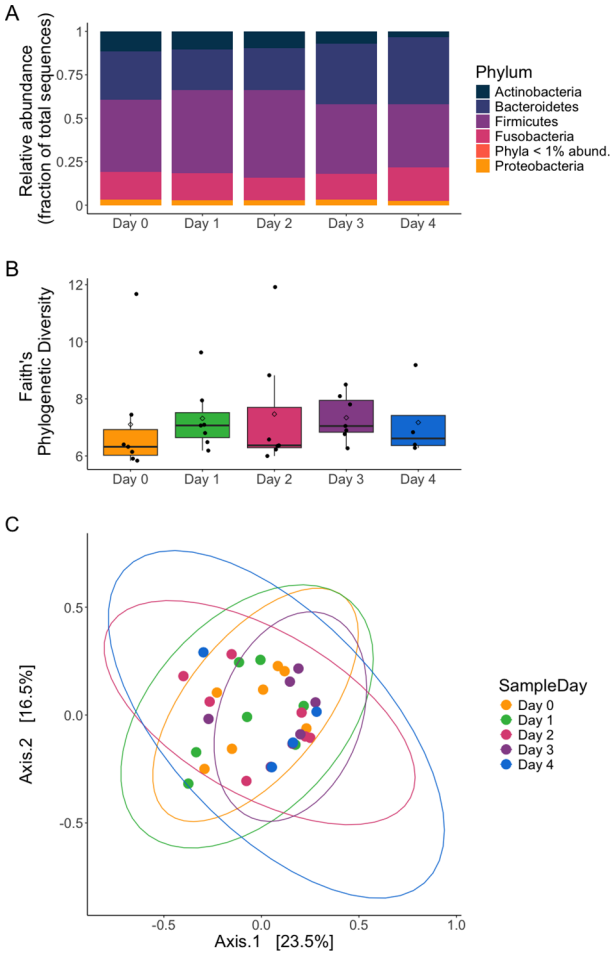

FIGURE 1
Namibian (NAM) cheetah fecal microbiota generally did not change from fresh sample day across any other sample day (colors) for **(A)** relative abundance (except for Actinobacteria), **(B)** alpha diversity (PD), or **(C)** beta diversity metrics. **(A)** Pooled relative abundance of dominant bacterial phyla in NAM cheetah feces by sample day (Days 0-3, n=8 samples each day; Day 4, n=4). Phyla with < 1% relative abundance were grouped together. Only Actinobacteria phylum were differentially abundant, with day 4 different from the other days. **(B)** Faith's phylogenetic diversity (PD) for NAM feces did not change across sample days **(C)** Bray-Curtis dissimilarity by sample day; no later days (Days 1-4) were different from Day 0 (fresh).

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similar across sample day groups (PERMDISP: Bray-Curtis p = 0. 754, Jaccard p = 0.257, unweighted Unifrac p =0.754).

3.2 Comparisons of fresh samples from two populations

In the comparison of fresh fecal samples from Namibia and the USA, we obtained a total of 50,800 high-quality sequences from 16 samples after rarefying by lowest sequence sum (3,176). There were 226 ASVs from six phyla including Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria, and Candidatus Saccharibacteria (Figure 2). None of the phyla were differentially abundant between the two populations (Wilcoxon rank test; all phyla p.adj > 0.62).

Analysis of microbial community revealed differences in ASV diversity and composition between the two populations. Within group diversity measures were higher in the Namibian samples in both SR and PD (ANOVA; SR: $F_{1,14}$ = 18.96, p = 0.0007 and PD: $F_{1,14}$ = 29.79, p = 0.00008; Figure 3). SR for NAM was 88.4 ± 12.6 vs USA 64.5 ± 9.06 and PD for NAM was 4.97 ± 0.47 vs USA 4.01 ± 0.17. Between group microbial composition also varied by population (PERMANOVA; Bray-Curtis Pseudo-$F_{1,14}$ = 5.1036, R^2 = 0.267, p = 0.002; Jaccard Pseudo-$F_{1,14}$ = 7.8492, R^2 = 0.359, p = 0.001; Unweighted UniFrac Pseudo-$F_{1,14}$ = 8.019, R^2 = 0.364, p = 0.002; Figure 4). Dispersion of the samples within a group (Population) around the centroid were determined to be similar (p > 0.05) across populations (PERMDISP: Bray-Curtis p = 0. 498, Jaccard p = 0.397, unweighted Unifrac p =0.465).

Overall, NAM samples had 104 ASVs not found in the USA samples, corresponding to 46% of the total number of unique 226 ASVs, while the USA had 54 unique ASVs (24%). The two populations shared 68 out of the total 226 ASVs (30%). When ASVs were weighted by abundance (number of sequences per ASV, where more abundant ASVs carry more weight), the proportion of total sequences (50,800 sequences) shared between the two populations increased to 69% (35,052 sequences from 68 ASVs). Whereas 20% of sequences (10,160 sequences from 104 ASVs) were unique to NAM samples alone, only 11% (5,588 sequences from 54 ASVs) were unique to USA samples.

Using LefSe LDA we found 26 specific taxa that were more common in either the NAM (21 taxa) or USA cheetahs (5 taxa; Figure 5A). Hierarchical clustering of samples based on abundance of these 26 taxa cleanly delineates NAM versus USA samples (Figure 5B). Taxa that were enriched in the Namibian samples belonged to three phyla, Actinobacteria (including *Coriobacteriaceae Collinsella*), Bacteroidetes (including *Porphyromonadacea Barnesiella* and *Prevotellacea Alloprevotella*), and Firmicutes (including *Erysipelotrichaceae Faecalitalea*, *Lachnospiracea Ruminococcus2*, *Peptococcaceae Peptococcus*, and *Ruminococcacea Clostridium IV*). The five taxa enriched in USA cheetahs were also found in the NAM cheetahs, but in lower amounts. By contrast, eight of the taxa present in the NAM cheetahs were missing completely from all of the USA samples (Figure 5B). These eight taxa were all members of Bacteroidetes (*Prevotellaceae, Alloprevotella*, *Porphyromonadaceae*, *Barnesiella*, and two unknown species in the *Alloprevotella* and *Barnesiella* genera) or Firmicutes (*Faecalitalea* and an unknown species in the *Faecalitalea* genus).

When looking at predictive functional analyses, metabolism was the highest predicted Level 1 KEGG Orthology (KO) category function of the microbiome, using PICRUST2 analyses in both populations (NAM 78.4% ± 0.01% and USA 78.6% ± 0.2%). None of the Level 1 KO categories differed between the two populations. For Level 2 KO category comparisons, USA samples had higher proportions of sequences that approached significance ($p.adj$ <0.1) and were associated with lipid metabolism, parasitic infectious disease, and carbohydrate metabolism (Supplementary Figure S3; Wilcoxon test with Benjamini Hochberg corrected p-values; lipid metabolism: effect = 1.29, overlap = 0.06, p.adj = 0.054; Parasitic infectious disease: effect = 1.06, overlap = 0.13, p.adj = 0.076; Carbohydrate metabolism: effect = 1.04, overlap = 0.11, p.adj = 0.09). However, the total proportion of sequences attributed to these categories for both groups was small. Among the most specific categories, Level 3, there were no pathways that differed between the two cheetah populations (see Supplementary Materials for model outputs for all three levels).

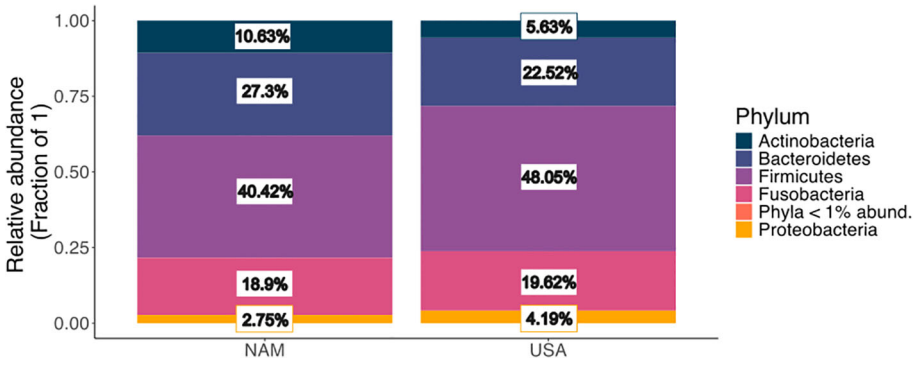

FIGURE 2
Pooled relative abundances (fraction of total sequences) of phyla for fresh fecals from *ex situ* NAM (n=8) and USA (n=8) cheetah populations. Phyla <1% abundant (Candidatus Saccharibacteria) for NAM and USA were too low to visually appear on plot but were 0% and 0.01%, respectively for each population.

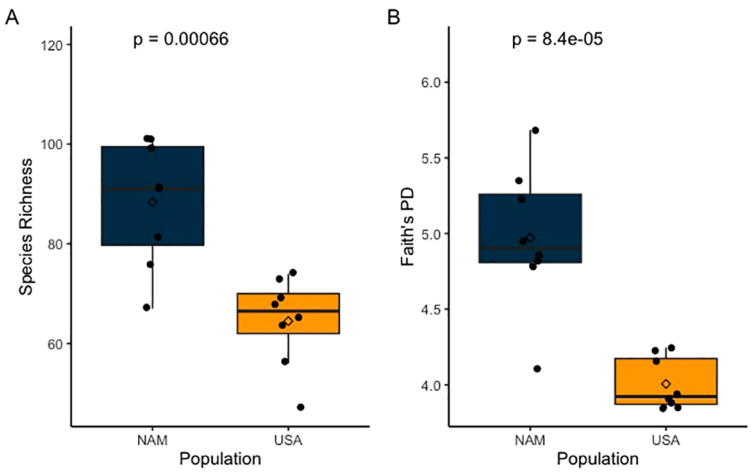

FIGURE 3
Box plots indicating fresh fecals from *ex situ* cheetahs in the USA (n = 8) exhibited lower **(A)** species richness (ANOVA, p = 0.00066) and **(B)** Faith's phylogenetic diversity (PD) (ANOVA, p = 0.00008) compared to fresh fecals (n=8) from *ex situ* NAM cheetahs.

4 Discussion

Wildlife gut microbiomes are of increasing interest because of their roles in overall organismal health. Fresh fecal samples are regarded as the gold standard for gut microbiome studies but are difficult to collect from wild animals. Here we performed two studies to better understand 1) how fecal microbiota change over time when exposed to the natural environment of the cheetah in Namibia and 2) how *ex situ* cheetahs in a USA facility compare to these *ex situ* cheetahs in a Namibian facility which are housed in habitat native to cheetahs and were assessed as a stand-in for true *in situ* populations. In aim one we found the cheetah fecal microbiota was stable in the dry Namibian environment for three days post-defecation which lengthens the time scientists can collect fecals in the wild under similar conditions. In aim two, there were strong differences in fecal microbiota between the fresh Namibian samples and the fresh USA samples that may have functional metabolic and disease-related consequences. Despite these differences we also found evidence for a conserved cheetah microbiome between the two populations.

4.1 Fecal microbial stability

In our first aim, we found that after three days of exposure to the dry Namibian environment, cheetah fecals were still similar in microbial diversity, composition, and structure to fresh fecals. However, Actinobacteria relative abundance began to change by

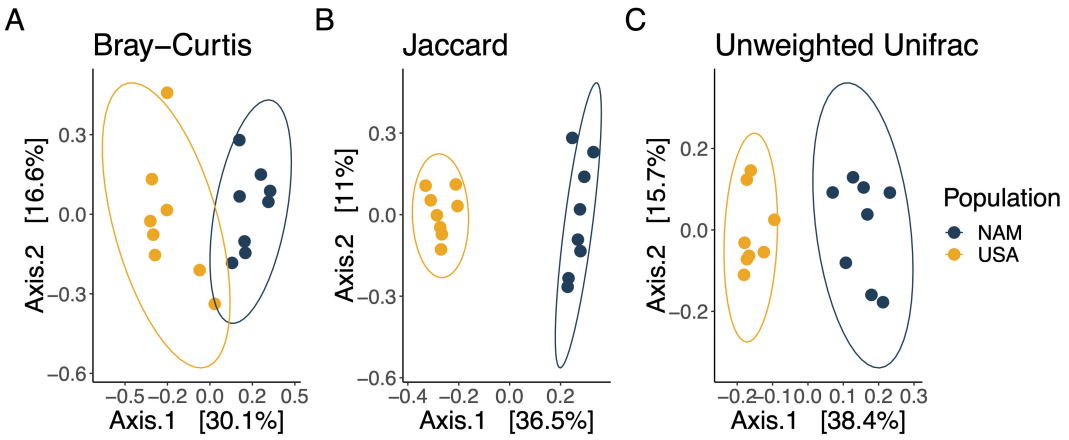

FIGURE 4
Principal coordinate analysis ordination plots show differences between the NAM and USA populations for **(A)** Bray-Curtis dissimilarity (p = 0.002), **(B)** Jaccard dissimilarity (p = 0.001), and **(C)** Unweighted UniFrac distances (p = 0.002) with 95% data ellipses by population. All p-values based on PERMANOVA models.

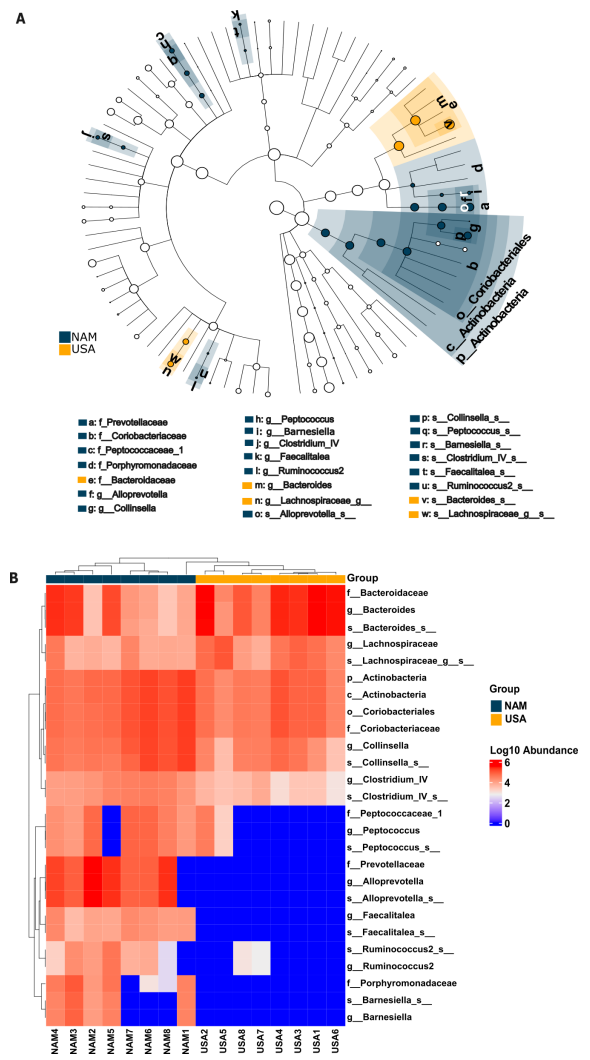

FIGURE 5
Linear Discriminant Analyses Effect Size (LDA LefSe) of bacterial taxa between NAM and USA *ex situ* cheetahs with LDA scores ≥ 4. Taxa are listed with a prefix indicating classification level (f = family, g = genus, s = species). Suffixes with blanks after the classification level ("_g:" or "_s:") indicates an ASV group that could not be confidently classified as a specific genus or species within the highest level listed (ex,. s_Lachnospiraceae_g:s: indicates an unannotated species in an unannotated genus within the family Lachnospiraceae). **(A)** Cladogram depicting phylogenetic relationships of enriched taxa by population (blue = NAM, yellow = USA, white = no difference between populations) where higher order classifications are labeled on the figure and lower (family, genus, species) are labeled with a letter and a corresponding taxon in the key; **(B)** Heatmap of enriched taxa in rows with individual cheetah samples as columns. Samples are clustered by both taxa and sample similarities. Log10 Abundance is indicated by color where darker red is highly abundant (6) and dark blue is absent (0).

Day 4. These patterns of compositional stability are similar to domestic cat samples in tubes at room temperature (Tal et al., 2017) but longer than previously reported in *ex situ* cheetah fecals sampled during a hot (max daily temperatures 27-33°C, Supplementary Table S2) and wet (max cumulative rainfall over a five day study period 2.1 cm, average max daily humidity 98.7%) summer season in Virginia, USA (Maly et al., 2024). In our previous US-based cheetah study, there were shifts in bacterial composition in abundance weighted and presence-absence metrics that occurred by Day 2 post-deposit. There was no precipitation during the sampling period for the current study as opposed to the US based study which experienced heavy rain during temporal sampling. The arid (0 cm of rainfall, average max daily humidity 40.9%) climate during the dry season in Namibia may aid in stabilizing the post-defecation shifts of the fecal microbiota. Similarly, giraffe fecal microbiota composition was stable in the dry Namibian environment until rain occurred before day five sampling in the series (Menke et al., 2015). It is important to note that fecals left in the experiment plot were covered with screens to prevent the fecals from being taken by larger insects (e.g., dung beetles), small animals, etc., which may have offered additional protection against microbial alterations from these or other large insects and animals (Wong et al., 2016). However, by collecting the middle core of the fecal not exposed to the environment directly, we hoped to mitigate these caveats as much as possible. Our sample size was limited due to the nature of working with non-model endangered animals and while our study may benefit from increased sample size to account for potential variation among individuals, our sample size was similar to studies of a similar nature (Menke et al., 2015; Tal et al., 2017; Lafferty et al., 2022; Wong et al., 2016). Together, our data suggest that researchers sampling cheetahs in Namibia during the dry season can collect up to 3-day-old fecal samples, as a proxy for studying gut microbiota. If collecting during a time of higher moisture (e.g., rain), however, based on our previous work, it is likely the collection window is closer to around 24 hours (Maly et al., 2024).

Physical characteristics of the NAM samples over time were similar to those previously reported in *ex situ* US samples (Maly et al., 2024). In brief, fresh samples were wet and shiny, very dense, pungent, and exhibited frequent insect activity (see Supplementary Figures). By Day 1, a crust started to form but the outside was still a bit tacky and the inside of the fecal still contained some soft feces and moisture. Day 2 feces had well-formed crusts that appeared dark and were beginning to dry out, even on the inside. Days 3 and 4, most fecals, especially those that were not well-formed logs, were very dark and very dry.

Finally, our study timeline terminated after four days post fresh, but it may be useful to sample for an extended period to identify changes beyond four days to aid in assessing the age of fecals from the field when the deposit event was not witnessed. We were unable to determine fecal degradation day age indicators with the current study because there were so few microbial differences across four days.

4.2 Comparisons of fresh samples from two populations

We compared fresh samples of the above Namibian project with fresh samples at a US facility from a previous study (Maly et al., 2024). NAM and USA samples consisted of the same main five Phyla previously reported for cheetahs (Wasimuddin et al., 2017; Menke et al., 2014; Maly et al., 2024). All five Phyla were similar in abundance across both populations, exhibiting greater proportions

of Firmicutes compared to Bacteroidetes. Though abundance data are not directly comparable between studies, it is noteworthy that the *ex situ* NAM and USA cheetahs in the current study had numerically lower abundances of Firmicutes [41.2% (time dataset NAM]/40.4% (combined dataset NAM) and 48.0% USA] compared to the wild cheetahs [56.2% (Menke et al., 2014) and 68.5% (Wasimuddin et al., 2017)] which corresponds to a trend seen in a study on bobcats, where those in zoos trended toward lower Firmicute abundance ($p < 0.1$) compared to wild bobcats (Eshar et al., 2019). Research in domestic cats shows Firmicutes were less abundant in obese compared to healthy weight individuals (Fischer et al., 2017; Ma et al., 2022). The individuals included in our study were not obese, however it is possible that *ex situ* cheetahs are less lean than their wild counterparts which may explain the lower Firmicute abundance, although other factors may be involved.

We discovered a high degree of variation in the fecal microbial diversity, composition, and structure between the two populations. We can assume the higher diversity of *ex situ* NAM fresh samples may be due to a variety of factors, including cheetah diet, demographics (age, sex, reproductive status), and environmental factors (climate and location). Since the scope of this study was not to define the effects of these differences, we did not control for them; however, some information appears relevant. Differences in food structure (minced vs whole prey mice) were responsible for significant differences in fermentation profiles in domestic cats, however they did not result in different alpha diversity estimates (D'Hooghe et al., 2024). The diet between the two facilities differed, with primarily donkey meat on the bone for the NAM vs commercial ground beef as the primary diet for USA; however, USA cheetahs were not fed solely minced commercial diet as they were given weekly whole prey items and bones. There is evidence that carcass diets contain higher amounts of non-digestible elements which obligate carnivores such as the cheetah, may have evolved to utilize as a source of dietary fiber (Depauw et al., 2013, 2012), and which may alter the gut microbiomes. Other important aspects that may influence the fecal microbiota include cheetah demographic data such as age (ranges, NAM: 3-14.5 years; USA: 1-6 years (Wasimuddin et al., 2017; Masuoka et al., 2017; Rojas et al., 2023), and sex (Wasimuddin et al., 2017)). Most evident of all, the two populations lived on different continents and the cheetahs and voided fecals were therefore exposed to different environmental conditions including but not limited to weather (Maly et al., 2024; Menke et al., 2015) and habitat (Gani et al., 2024) that likely also influenced the microbiota. Thus, a number of variables could be responsible for the differences we find between populations, and future studies comparing many populations are needed to determine their relative impact on cheetah microbiota.

Bacterial communities differed between the two populations in all measures of diversity and composition examined, with 26 taxa that were enriched in either NAM or USA samples. Interestingly, eight taxa were enriched in NAM samples but absent in the USA samples. A few of these taxa belong to the family *Prevotellaceae*, some of which are known carbohydrate and protein fermenters in humans (De Filippo et al., 2010; Aguirre et al., 2016). *Prevotellaceae* are part of the healthy domestic cat microbiome (Ganz et al., 2022). Domestic cats fed whole mice had enriched *Prevotellaceae* compared to those fed minced mice and were associated with food structure differences between minced and whole prey (D'Hooghe et al., 2024). *Alloprevotella*, a genus within *Prevotellaceae*, were enriched in NAM samples and absent in USA samples, and some members of this genus can hydrolyze gelatin from collagen and produce short chain fatty acid acetic acid and succinic acid (which can be metabolized into another SCFA, propionic acid) (Downes et al., 2013; Leaver et al., 1956). Another taxon enriched in the Namibian samples, *Barnesiella*, has been associated with reduced inflammation in asthma (Zhang et al., 2021) and allergic reactions (Vital et al., 2015). Interestingly, *Barnesiella* was also found to restrict the growth of an antibiotic-resistant bacteria in mice and humans (Ubeda et al., 2013). The presence of these microbes may play a role in the fewer incidences of symptoms related to GI inflammation (including gastritis) reported in these Namibian cheetahs Mangiaterra et al. (2022), but further research is needed to address their roles in the cheetah GI tract. Interestingly, we found 68 bacterial ASVs (30% of the microbiome) that were shared between the two populations, suggesting that while environment, diet, and demographics may impact the gut microbiota, there is still a conserved cheetah microbiome that likely plays critical functions in cheetah physiology regardless of associated extrinsic and intrinsic factors. Of those shared, ASV1, a Fusobacterium, was the most abundant and is an anaerobic protein fermenter (Mead, 1971) commonly found in domestic cat microbiomes, especially those consuming raw meat diets (Butowski et al., 2019).

PICRUSt2 analyses showed approximately 78% of all sequences in both populations were associated with metabolism. This was expected given the microbes reside in the gut where host and microbial metabolic processes take place to utilize digestive material. Across all levels and categories, only three KO pathways (all Level 2) approached significance between the two populations (Supplementary Tables 3-5). Two of these pathways were types of metabolism. Carbohydrate metabolism is likely higher in the USA samples due to the beet pulp in the commercial ground beef diet. Lipid metabolism was also higher in the USA samples. In domestic cats, multiple types of Level 3 lipid metabolism pathways were higher in cats with acute diarrhea (Bai et al., 2023). Two cheetahs in the USA population were experiencing acute diarrhea at the time of collection, which may at least partially explain these differences. Lastly, the finding that the USA cheetahs had higher proportions of bacterial pathways related to parasite infection was unexpected. The USA cheetahs received monthly parasitic preventatives including Ivermectin for helminths and Frontline (Boehringer Ingelheim, Ingelheim, Germany) for ticks and fleas, so their parasite load should be relatively low. In contrast, NAM cheetahs were regularly monitored for parasites and prescribed treatment only when necessary. It is important to note, that these sequences are based on predicted gene content of the bacteria found in the samples from the two populations and not direct sampling for parasites. These differences may be due to the presence of bacteria that simply carry genes that are known to be involved in pathways of parasitic infectious disease but are not a direct indication of parasite presence. In general, these PICRUSt2 data provide an estimation of the bacterial functional production in the cheetah gut microbiome.

Based on these data, we recommend future studies focus on the microbiome, transcriptome, and metabolome of managed and wild cheetahs to better understand their relationship with host genetics and physiology, diet, and environment. Our main recommendation is to focus on the relationship with diet, particularly in managed cheetahs. We suggest studies to manipulate the cheetah diet in a controlled setting to determine whether the inclusion of non-digestible animal fibers alter the microbiome and metabolome, and at what proportion these dietary fibers produce changes in the microbiota. There are multiple prey species options for carnivores in managed care, but in many cases single-species origin diets are standard. It would be of interest to know if the diversity of animal species in the diet influences the microbiome, metabolome, or susceptibility to GI disease. Additionally, to improve our knowledge on the etiology of GI disease in cheetahs, we recommend an initial direct comparison of cheetahs with and without chronic gastritis. Further, we suggest controlled longitudinal studies on cheetahs starting at cub stage and following them over the course of their life. If animals in the longitudinal studies are fed diets with different levels of non-digestible animal fiber, they should help determine if fiber levels lead to differences in the microbiome and metabolome that may in turn offer protective effects against GI disease.

In conclusion, our data suggest cheetah fecals from the dry season (no rain) in Namibia are acceptable for microbiota collection for up to three days post defecation. This is longer than in our previous study where cheetah fecals exposed to rain and more humid conditions were only stable for 24 hours. These data suggest moisture is an important component to consider for fecal collections when utilized as a proxy for the gut microbiome. We hypothesize these patterns will apply to other members of the *Felidae* family, and perhaps even other large obligate carnivores under the same climatic conditions, but we recommend additional time series studies to confirm these predictions in new species or environments. These data will provide greater access to cheetah and other large carnivore fecals and reduce costs for sampling efforts, offering an extended period for non-invasive fecal collection. In the second aim we identified differences in fecal microbiota diversity, composition, enriched taxa, and predictive functional relevance between two *ex situ* cheetah populations. While we were unable to control for or determine which factors were responsible, it does provide evidence that warrants further investigation into managed cheetah gut microbiomes and diet. Many of the differentially abundant taxa between the two populations have known clinical relevance in the gastrointestinal tract, being key taxa to examine in relation to GI health that is negatively influencing captive cheetah husbandry and breeding success. We recommend future studies to assess the functional effects of *ex situ* diet type on cheetah microbiomes and metabolomes to improve cheetah welfare and breeding program success.

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: https://www.ncbi.nlm.nih.gov/bioproject/, BioProject PRJNA1160820. The data and code for aim one are available on Dryad: https://doi.org/10.5061/dryad.sn02v6xdq, and for aim two: https://doi.org/10.5061/dryad.jm63xsjm5. Additionally, the code and data are available on GitHub: https://github.com/Malytherin/CheetahFecalStability_Namibia and https://github.com/Malytherin/Comparing.Fresh.Fecals_NAMvUSA.

Ethics statement

Ethical approval was not required for the studies involving animals in accordance with the local legislation and institutional requirements because collection of non-invasive fecal material is exempt from IACUC approval. Sample collection was performed under the permit number 2018051701 at the Cheetah Conservation Fund (Namibian-based Research Institute RCIV00122018). For USA cheetahs, written informed consent was obtained from the owners for the participation of their animals in this study. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MAM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. RR: Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing, Formal analysis, Resources. MK: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. AS-K: Methodology, Writing – review & editing, Conceptualization, Project administration, Resources, Supervision. MM: Writing – review & editing, Methodology, Data curation, Formal analysis, Visualization. LM: Project administration, Resources, Supervision, Writing – review & editing. MB: Funding acquisition, Supervision, Writing – review & editing. CM-W: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing, Data curation, Validation. AC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2024.1503026/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Mesh window screens were placed over top of fecals to prevent dung beetles and other animals from removing them from the plot. **(A)** Window screen placed over fecal sample; **(B)** Screen mesh from the windowpane still allowed sun, precipitation, and small bug activity but kept out larger insects and animals; **(C)** Experimental plot for degrading fecal samples.

SUPPLEMENTARY FIGURE 2
(A) Alpha and **(B, C)** beta diversity measures for NAM fecal samples across sample day (colors). **(A)** Species richness (SR) did not differ by sample day (GLMM: x^2 = 0.6343, df = 4, p = 0.959). **(B)** Jaccard and **(C)** unweighted Unifrac distances did not differ by sample day (PERMANOVA Sample Day: Jaccard Pseudo-$F_{4,22}$ = 0.4545, R^2 = 0.07632, p = 0.24, UniFrac Pseudo-$F_{4,22}$ = 0.4595, R^2 = 0.07711, p = 0.099).

SUPPLEMENTARY FIGURE 3
Proportions of sequences for Level 2 KEGG Ortholog predicted functional pathways **(A)** lipid metabolism (Wilcoxon rank test, effect = 1.29, overlap = 0.06, p.adj = 0.54), **(B)** parasitic infectious disease (Wilcoxon rank test, effect = 1.06, overlap = 0.13, p.adj = 0.076), and **(C)** carbohydrate metabolism (effect = 1.04, overlap = 0.11, p.adj = 0.09). Color indicates population where blue is Namibian (NAM) and yellow is US (USA) cheetahs. Outliers are indicated by points.

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Embryo reintroduction to enhance genetic diversity in a Marsh deer population: first attempt, outcomes, challenges, and future perspectives

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Introduction: The loss of genetic diversity is a critical factor in the extinction process, exacerbated by anthropogenic pressures and demographic stochasticity, particularly in small populations. Traditional population restoration methods, such as individual translocation and ecological corridors, present challenges, including high costs, adaptation difficulties, pathogen introduction, and outbreeding risks. Embryo reintroduction has emerged as a potential strategy for genetic rescue. This study aimed to evaluate the feasibility of embryo transfer as a genetic rescue tool in the marsh deer (*Blastocerus dichotomus*), a species classified as Vulnerable by the IUCN.

Methods: Following the construction of the Sérgio Motta Hydroelectric Power Plant (UHSM) in 1998, a population of over 1,000 marsh deer was impacted, leading to the capture of 93 individuals for *ex situ* (82) and *in situ* (11) conservation efforts. Between 1998 and 2001, an experimental reintroduction program established a new population in a 2,000-hectare wetland near the Jataí Ecological Station (EEJ) in Luis Antônio, São Paulo, Brazil. Over time, this population reached a carrying capacity of 25 individuals but experienced genetic diversity loss. To address this, we conducted an embryo transfer experiment using a female from the reintroduced population as a receipt for embryos from the captive population. The female, captured during late pregnancy, was subjected to estrous cycle synchronization for embryo transfer after giving birth and being apart from its fawn. Two embryos from a captive population were implanted, and the female was re-released after 10 days. Monitoring was conducted via radio transmitter collar (GPS–GSM) and helicopter tracking.

Results: The female did not give birth to the implanted embryos but was recaptured nine months later for an ultrasonographic evaluation, which indicated a six-month pregnancy. This suggests that the implanted embryos were lost early in gestation, but natural fertilization occurred approximately three months post-release.

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Discussion: This study demonstrates the technical feasibility of embryo reintroduction as a genetic rescue strategy, even though pregnancy was not carried to term. The ability to capture, temporarily hold, and successfully reintroduce a free-ranging female suggests minimal disruption to natural behaviors. Future improvements in embryo quality, hormonal protocols, and pregnancy confirmation prior to release could enhance the success rate of this method. Embryo reintroduction presents a promising alternative to traditional reintroduction methods, offering a novel approach to mitigating genetic risks in small, isolated populations.

KEYWORDS

***Blastocerus dichotomus*, conservation program, assisted reproductive techniques, genetics, ecology**

1 Introduction

Reintroduction programs are crucial for conserving species at risk of extinction, but their success depends on several factors, with genetic concerns playing a central role. A major challenge in reintroductions is the potential loss of genetic diversity, often exacerbated by the small number of individuals typically involved. This can lead to inbreeding and the founder effect. Inbreeding may lead to inbreeding depression and, along with genetic drift, results in the loss of genetic variability, negatively affecting fertility, survival, and overall population viability (Falconer, 1989). The founder effect occurs when a small subset of individuals is separated from the larger population, carrying only a fraction of the genetic diversity, which over time leads to allele loss, reduced heterozygosity, and decreased population fitness (Shorrock, 1980). Genetic and demographic founder effects can have long-term consequences for the colonizing populations, compounding the risks of inbreeding depression and hindering the population's recovery (Szűcs et al., 2017). These genetic challenges are particularly concerning for species like the marsh deer (*Blastocerus dichotomus*), given its restricted distribution and the risk of future genetic bottlenecks, that threaten its survival.

The marsh deer is the largest deer species in South America, reaching up to 100 kg for females and 130 kg for males (Piovezan et al., 2010). It inhabits floodplains and wetland areas, historically ranging from the southern Amazon Rainforest to northeastern Argentina (Piovezan et al., 2010). Several populations are currently at risk of extinction due to poaching, livestock disease exposure, floodplain drainage for agricultural expansion, and the construction of hydroelectric dams; therefore, the species is categorized as vulnerable by the IUCN (Szabó et al., 2003, 2007; Piovezan et al., 2010; Duarte et al., 2016). These factors may cause severe population declines and reduced genetic variability due to inbreeding and genetic drift, impairing reproductive performance and limiting adaptive potential (Roldan et al., 2006; Duarte et al., 2012).

In the early 1990s, following the construction of the Três Irmãos Hydroelectric Power Plant on the Tietê River by Companhia Energética de São Paulo (CESP), 45 marsh deer were rescued and placed in captivity. This led to the creation of the Marsh Deer Captive Management Program, currently managed by Tijoá Energia. The program was operated at the Marsh Deer Conservation Center (CCCP) in Promissão, São Paulo, and the Ilha Solteira Zoo in Ilha Solteira, São Paulo. Then within the same decade, the construction of the Porto Primavera Hydroelectric Power Plant (UHSM) on the Paraná River in 1998 brought the last significant population of marsh deer in São Paulo to the brink of extinction. In response, the Porto Primavera Marsh Deer Project was established to study and conserve the affected population through *ex situ* conservation efforts, including the reintroduction of animals into remnant floodplain areas in São Paulo (Zanetti and Duarte, 2008).

In late 1998, the Marsh Deer Conservation Program was initiated by researchers from the Deer Research and Conservation Center (NUPECCE) at São Paulo State University (UNESP-Jaboticabal) to study the impact of the UHSM on the local marsh deer population. The project aims to conserve the species through *ex situ* programs to preserve genetic diversity and provide animals for reintroduction efforts, such as those performed at the Jataí Ecological Station (EEJ) in São Paulo (Figueira et al., 2005; Zanetti and Duarte, 2008). Despite initial challenges, a reintroduced population at the EEJ was established from eight individuals (three males and five females) between 1998 and 2001. Then, between July 2004 and June 2007, individuals from this population were captured for transmitter ear-tag placement, biological material collection, and monitoring, estimating the population size between 20 and 25 individuals (Ferreira, 2011). Although the loss of genetic diversity and inbreeding were not yet concerns, simulations indicated that long-term genetic diversity could only be maintained by introducing new individuals every four generations (Ferreira, 2011).

Given the challenges of translocation efforts, including high mortality rates and diseases risks (Warne and Chaber, 2023), embryo translocation (ET) offers a promising alternative for

genetic management and disease risk reduction in reintroduced populations (Rola et al., 2021, 2023). However, to fully utilize ET for species conservation, it is crucial to develop effective superovulation protocols for generating embryos from captive animals. Reproductive studies on marsh deer provide important insights into their breeding biology, which can help to develop and adapt these protocols. Female marsh deer reach sexual maturity at around two years of age, with an average estrous cycle of 21.3 ± 1.3 days. They are polystric and uniparous, with an average pregnancy length of 253 ± 4 days and no reproductive seasonality, as evidenced by postpartum estrus (Polegato et al., 2018). Males display individual variation in antler cycles, with minimal synchronization and no apparent photoperiod influence, indicating year-round fertility (Ramos, 2004; Pereira, 2010). These basic reproductive characteristics are crucial for optimizing ET techniques and enhancing genetic management in conservation programs (Comizzoli, 2020).

Despite progress, the program faces challenges in maintaining optimal population sizes and genetic diversity, particularly concerning male management and institution capacity (Ferreira, 2011). The original population of 82 animals distributed in 18 Brazilian institutions had decreased to 67 individuals distributed in 12 institutions by 2020 (considering only the UHSM population). These challenges highlight the need for a genomic embryo bank to enhance genetic management and ensure the long-term success of the conservation program (Comizzoli, 2020; Rola et al., 2021).

This study aimed to bridge captive populations with wild counterparts by collecting embryos from the Brazilian captive population to establish a cryogenic bank for the species and developing an embryo reintroduction technique to assist the wild EEJ population.

2 Materials and methods

2.1 Animals

Our captivity experimental group to develop an embryo bank consisted of ten adult females and four fertile males of marsh deer from the Brazilian captive populations, both of the populations of Três Irmãos Hydroelectric Power Plant and the Porto Primavera Hydroelectric Power Plant (UHSM). This group was subdivided into three different treatments (n = 3, Table 1), and one free-living female captured for the embryo transfer. The animals were maintained in two installations. The first one was the Marsh Deer Conservation Center (Centro de Conservação do Cervo-do-Pantanal, CCCP), Promissão, São Paulo, Brazil. Animals were maintained individually in paddocks (40 m x 100 m) and were fed with a diet consisting of a pelleted ration (Essence Traditional – Presence®), approximately 500 g/deer/day of fresh abobra (*Abobra tenuifolia*), 250 g/deer/day of fresh roselle (*Hibiscus sabdariffa*) and water *ad libitum*. The second one was the Deer Research and Conservation Center (Núcleo de Pesquisa e Conservação de Cervídeos, NUPECCE), São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil. Animals were maintained individually in stalls (4 m x 4 m) and were placed in paddocks (20 m x 60 m) when estrus detection was performed. Animals were fed with a diet consisting of a pelleted ration (Equi Tech 12MA – Presence®), approximately 2 kg/deer/day of fresh perennial soybean (*Neonotonia wightii*), ramie (*Boehmeria nívea*), or mulberry branches (*Morus alba*), and water *ad libitum*. All animals maintained auditory and olfactory contact with conspecific males and females and were exposed to normal fluctuations in the photoperiod. All animal procedures were approved by the Ethics Committee on Animal Use (CEUA) of the School of Agricultural and Veterinarian Sciences from São Paulo State

TABLE 1 Characteristics of 13 deer of the species *Blastocerus dichotomus* that comprised the experimental groups.

Deer	Age (years)	Reproductive history	Treatment	Origin
F358	2 (08/12/2011)	Nulliparous	A	CCCP
F375	4 (04/07/2012)	Nulliparous		CCCP
F381	2 (03/09/2012)	Nulliparous		CCCP
M372	2 (19/05/2012)	Fertile		CCCP
M373	2 (23/05/2012)	Fertile	A & B	CCCP
F210	12 (29/11/2001)	Multiparous	B	CCCP
F261	11 (24/11/2002)	Nulliparous		CCCP
F344	3(08/03/2011)	Primiparous		CCCP
M325	5 (11/02/2009)	Fertile		CCCP
F61	15 (16/05/2000)	Multiparous	C	NUPECCE
F315	7 (14/07/2008)	Multiparous		NUPECCE
F366	3 (04/04/2012)	Nulliparous		NUPECCE
M292	5 (~/10/2009)	Fertile		NUPECCE
NN	Unknown	Multiparous	No treatment/embryo transfer	EEJ

CCCP, Marsh Deer Conservation Center; NUPECCE, Deer Research and Conservation Center; EEJ, Jataí Ecological Station; NN, No number.

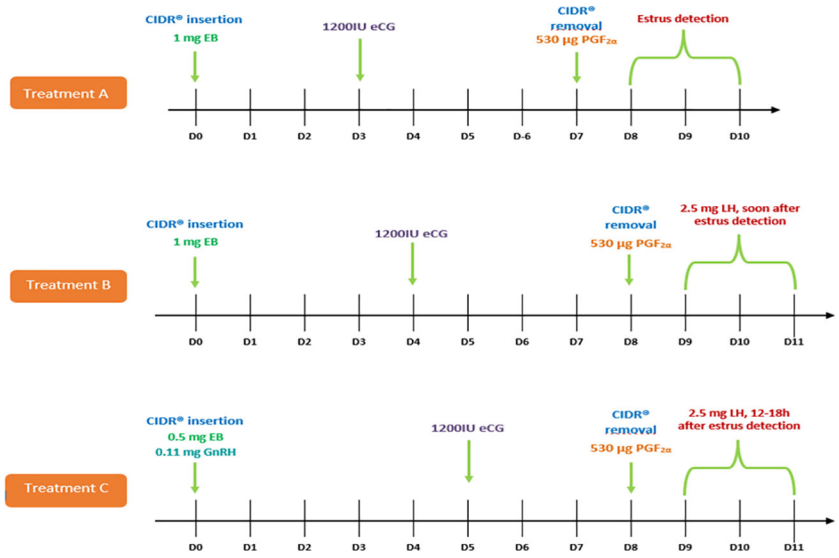

FIGURE 1
Treatments for superovulation and ovulation induction in *Blastocerus dichotomus*. Treatment A: CIDR® (7 days) + 1 mg EB (D0) + 1,200IU eCG (D3) + 530 µl $PGF_{2\alpha}$ (D7); Treatment B: CIDR® (8 days) + 1 mg EB (D0) + 1,200IU eCG (D4) + 530 µl $PGF_{2\alpha}$ (D8) + 2.5 mg LH soon after estrus detection; Treatment C: CIDR® (8 days) + 0.5 mg EB + 0.11 mg GnRH (D0) + 1,200IU eCG (D5) + 530 µl $PGF_{2\alpha}$ (D8) + 2.5 mg LH between 12-18 h after estrus detection.

University (approval N° 13721/15), in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2 Hormonal treatment and estrus detection for *in vivo* embryo production

We conducted three equine chorionic gonadotropin (eCG-based) superovulation (SOV) protocols for *B. dichotomus* (Figure 1). An adaptative system was adopted, and adjustments were made after each treatment until a superovulatory response was obtained. The treatments did not take into consideration the seasons of the year, because marsh deer hinds are non-seasonal breeders (Polegato et al., 2018).

Treatment A. In June 2014, three adult females (n = 3) (weighing 70.0 to 80.0 kg) and two males (weighing 90.0 to 100.0 kg) (Table 1) were housed in the CCCP. On a random day of the estrous cycle (D0), females received a single intravaginal device containing 0.33 g of progesterone (CIDR®-type T; Pfizer®; USA) for seven days. Still on D0, they received 1 mg of estradiol benzoate (EB) (Sincrodiol®; Ourofino Saúde Animal Ltda., Brazil) by intramuscular (i.m.) injection. They also received 1, 200 IU eCG (Folligon®; Intervet® Schering-Plough Animal Health, Brazil) by i.m. injection on day 3 following CIDR® insertion, and 530 µg of cloprostenol (PGF2α; Ciosin®; Intervet® Schering-Plough Animal Health, Brazil) by i.m. injection the day CIDR® was removed (D7).

Treatment B. In July 2014, three adult females (n = 3) (weighing 80.0 to 95.0 kg) and two males (weighing 90.0 to 110.0 kg) (Table 1) were housed in the CCCP. On a random day of the estrous cycle (D0), females received a CIDR® for eight days. Still on D0, they received 1 mg of EB by i.m. injection. They also received 1,200 IU of eCG by i.m. injection on day 4 following CIDR® insertion and 530 µg of PGF2α by i.m. injection the day the device was removed (D8). Soon after estrus detection, they received 2.5 mg of luteinizing hormone swine pituitary (LH) (LUTROPIN®-V - Bioniche® - Canada) by i.m. injection.

Treatment C. Between May and June 2015, three adult females (n = 3) (weighing 70.0 to 95.0 kg) and one male (weighing 110.0 kg) (Table 1) were housed in NUPECCE. On a random day of the estrous cycle (D0), females received a CIDR® for eight days, 0.5 mg of EB and 0.11 mg of gonadorelin diacetate tetrahydrate (GnRH) (Cystorelin®; Merial®, USA) by i.m. injection. They also received 1,200 IU of eCG by i.m. injection on day 5 following CIDR® insertion and 530 µg of PGF2α by i.m. injection the day the device was removed (D8). Between 12 and 18 h after estrus detection, they also received 2.5 mg of LH by i.m. injection.

CIDR® insertion and removal, as well as EB and PGF2α applications were performed during chemical immobilization with an association between 1 mg/kg of azaperone (Stresnil®, Janssen Pharmaceutica, Bélgica) and 0.5 mg/kg of xilazine hydrochloride (Sedomin®, König Brazil Ltda., Brazil) (Carregaro et al., 2019) i.m. dart injection, that was applied at a distance by a blowgun. The eCG application was also performed by i.m. dart injection. In treatment C, the eCG and PGF2α applications, as well as CIDR® removal were carried out with the aid of a box with a small window to restrict and manipulate the animals (Duarte et al., 2010).

Behavioral estrus was defined as the period in which females permitted mating (Pereira et al., 2006) and was determined by grouping a female with a male in the paddock (10 – 20 minutes

every 12 h) from one day after CIDR® removal, until the moment that the female no longer accepted copulation (end of estrus).

2.3 Assessment of ovarian stimulation and embryo collection

SOV response was evaluated by counting *corpus luteum* (CL) on both ovaries. Total follicular stimulus was evaluated by counting CL and persistent follicles (large anovulatory follicles) (Blanco et al., 2003). Counting was performed 8 days after the first mating by ventral midline laparotomy. Thus, females with two or more CL were classified as superovulated (Zanetti et al., 2014). For surgical procedure, females were submitted to food and water fasting for 24 hours. Chemical restraint was done with a pharmacological combination of 7.0 mg/kg of ketamine hydrochloride and 1 mg/kg of xylazine hydrochloride at a distance by a dart gun (JM-standard, Dan-Inject) (Favoretto et al., 2012). Then, intubation was performed with an endotracheal catheter n° 9 and maintained under inhalation anesthesia with isoflurane (Generic Isoflurane - Instituto Biochimico Ind. Farm, Ltda. - Brazil). Cannulation of the cephalic vein was performed for the administration of fluid therapy at a rate of 10 ml/kg/h with 0.9% NaCl solution. Embryo collection was performed by oviduct and uterine flushing (Duarte and Garcia, 1995). The flushing medium used for embryo recovery was phosphate buffered saline (PBS) solution (Nutricell Nutrientes Celulares® - Campinas - Brazil) associated with 1% fetal bovine serum (FBS) (Nutricell Nutrientes Celulares® - Campinas - Brazil), heated to 37°C. At the end of the procedure, the surgical wound was sutured with nylon thread 2.0. In the immediate postoperative period, hinds received 40,000 IU/Kg of benzathine penicillin i.m. and 500 mg of phenylbutazone, i.v. They also received an i.m. application of 530 μg of PGF2α to induce luteolysis of the multiple CL and, eventually, prevent the implantation of embryos that remained after collection.

2.4 Embryo evaluation and vitrification

For embryo evaluation, flushing medium collected was analyzed using a stereo microscope at 10x. The collected embryos were washed in PBS plus 10% FBS for removal of cell debris and classified (with 50x magnification) according to the developmental stage (number of cells and morphology) and structural integrity, following the standards used for bovine embryos (Stringfellow and Seidel, 1999). All embryos were vitrified using commercial medium (Bioclone, Jaboticabal, São Paulo), stored in labeled vitrification straws, and subsequently preserved in liquid nitrogen.

2.5 Capture of free-living female from Jataí Ecological Station

In order to perform the embryo transfer into the reintroduced population at the Jataí Ecological Station, Luis Antônio, São Paulo, one adult female (weight = 84 kg) was captured and brought into captivity during July 2015. The animal was captured using the 'Bulldogging' method (Duarte, 2008), with the assistance of a Robinson 44 helicopter. After physical restraint, chemical sedation was administered via i.v. injection with a pharmacological combination of 0.05 mg/kg acepromazine, 5 mg/kg ketamine, and 0.5 mg/kg midazolam. A transrectal ultrasound examination (linear transducer, 7.5 MHz) was then conducted to confirm pregnancy and estimate gestational age (Zanetti et al., 2010a) (Figure 2). Following the decision to remove the animal from its original location and transport it to NUPECCE for embryo transfer procedures, the animal was airlifted by helicopter to the outer edge of the floodplain, where a vehicle was stationed to transport it to NUPECCE. The female was kept at the NUPECCE facilities with minimal handling to avoid its adapting to captivity. As a result, human contact was highly restricted.

FIGURE 2
The animal was physically restrained after capture, and a transrectal ultrasound was performed, showing the fetus in an advanced stage of development.

2.6 Synchronization protocol and embryo transfer into a wild female

One month after parturition, late October 2015, and considering the postpartum estrus in the species (Polegato et al., 2018), the wild female marsh deer was synchronized for embryo transfer. On a random day (D-8) of the estrous cycle the females received a CIDR® for eight days and an i.m. injection of 0.5 mg of EB. On the day of CIDR removal (D0), i.m injections of 530 μg of PGF2α and 450 IU of eCG were administered (Zanetti et al., 2009; modified).

Nine days after CIDR removal (D9), chemical restraint was administered, and the female underwent a laparoscopic procedure to assess the presence of the CL (Zanetti et al., 2010b; Zanetti and Duarte, 2012) (Figure 3A), followed by embryo transfer (Figure 3B). At the end of the procedure, the incisions were sutured, and the female received an i.m. injection of 40,000 IU/kg of benzathine penicillin. Before recovery from anesthesia, the animal was fitted with a Wild CellMG® radio transmitter collar (Lotek Wireless®, Canada - pre-programmed to send the animal's geographic coordinates via GSM). The collar weighed 950 g, which corresponds to 1.13% of the animal's body weight.

2.7 Female reintroduction into the wild and monitoring

Ten days after embryo transfer, in November 2015, the female was anesthetized and transported to its natural habitat, into the EEJ, in a transport box. Monitoring of the female after release was conducted using the GPS – GSM system of the radio transmitter collar. Two types of sampling were scheduled: (1) continuous location collection every hour to obtain information on the animal's area use and survival, and (2) intensive sampling every 15 minutes for 3 days following release and for another 4 days throughout gestation (one day per week) to track the animal's exact movements. We analyzed data from the first 2 months of monitoring to investigate the release process. During this period the GPS data resulted in 1514 localities collected from which 1427 (94%) were with high quality coordinates (DOP<10) and were used for area and movements estimations. Information was sent by the GSM-collar every 5 locations collected, thus resulting in a daily real-time monitoring, which was also used to recapture procedures. Nine months after the reintroduction of the female, a recapture was scheduled, in August 2016, with the expectation of finding female with a fawn at its side. The same capture protocol previously described was followed, with the help of the coordinates of its recent location transmitted via GSM.

2.8 Statistical analysis

Descriptive statistics, including the mean and standard deviation, were calculated for the number of CL, anovulatory follicles (AF), and total follicular stimulus (TFS) across treatments. Data normality was assessed using the Shapiro-Wilk test. For non-normal data (Treatment B), the Friedman rank test was applied, while data from Treatments A and C were analyzed using ANOVA. *Post-hoc* comparisons were conducted using Tukey's HSD or equivalent pairwise tests, depending on data distribution. A significance level of $p < 0.05$ was set for all analyses.

3 Results

3.1 Superovulation protocols and embryo recovery

Results for SOV response are presented in Table 2. For Treatment A, all females displayed behavioral signs of estrus, with subsequent mating with males after CIDR® removal, 61.33 ± 7.06 h

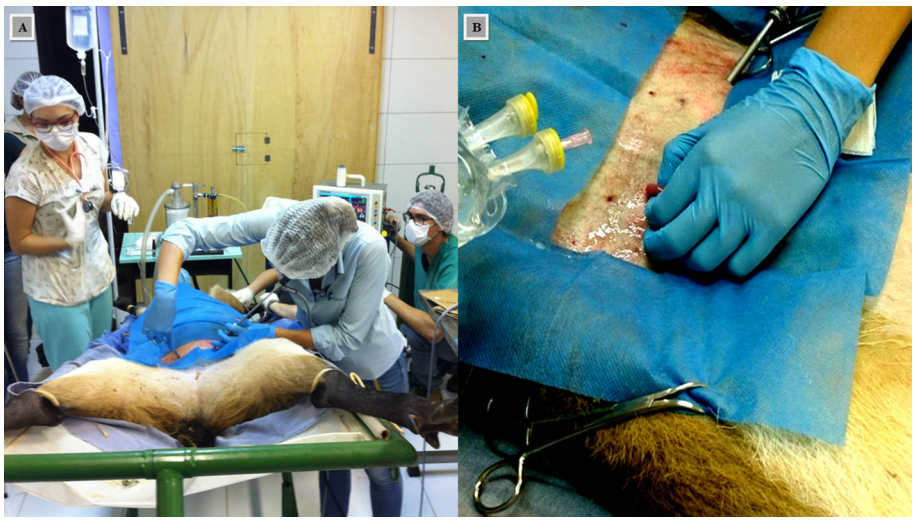

FIGURE 3
Embryo transfer surgery. **(A)** Exploratory laparoscopy to confirm the presence of a *corpus luteum*. **(B)** Exposure of the uterus for embryo inoculation.

10.3389/fcosc.2025.1537563

TABLE 2 Data of the estrus and ovarian response of nine hinds of the species *Blastocerus dichotomus*, submitted to three different superovulation treatments.

	Hind	Heat (h*)	Mating	Buck	CL	AF	TFS**	Embryo
Treatment A								
CIDR ovis + 1mg Sincrodiol (D0); 1,200 IU Folligon (D3); CIDR removal + 0.53mg Ciosin (D7)	F375	Yes (64h)	Yes	M372	01	07	08	00
	F358	Yes (72h)	Yes	M372	00	04	04	00
	F381	Yes (48h)	Yes	M373	01	03	04	01[a]
	Mean				0.67[A]	4.67[A]	5.33[A]	–
	Standard deviation				0.58	2.08	2.31	–
Treatment B								
CIDR ovis + 1 mg Sincrodiol (D0); 1,200 IU Folligon (D4); CIDR removal + 0.53 mg Ciosin (D8). 2.5 mg Lutropin-V (immediately after heat detection).	F210	No	–	–	–	–	–	–
	F261	Yes (64h)	Yes	M325	00	02	02	00
	F344	Yes (42h)	Yes	M373	02	00	02	01[b]
	Mean				1.00[A]	1.00[A]	2.00[B]	–
	Standard deviation				1.41	1.41	0.00	–
Treatment C								
CIDR ovis + 0.5 mg Sincrodiol + 0.11 mg Cystorelin (D0); 1,200 IU Folligon (D5); CIDR removal + 0.53 mg Ciosin (D8). 2.5 mg Lutropin-V (16 hours after heat detection).	F315	Yes (32h)	Yes	M292	10	00	10	02[a]
	F61	Yes (24h)	Yes	M292	03	01	04	02[b]
	F366	Yes (40h)	Yes	M292	11	05	16	05[c]
	Mean				8.00[B]	2.00[B]	10.00[C]	3.00
	Standard deviation				4.36	2.65	6.00	1.73

*Period between CIDR® removal and estrus detection.
**Sum of the number of corpora lutea and the number of anovulatory follicles.
[a]Embryo developmental arrest.
[b]Degenerate embryos.
[c]2 viable embryos, 1 regular embryo and 2 degenerate embryos.
CL, *Corpus luteum*; AF, anovulatory follicles; TFS, total follicular stimulus.
Superscript letters indicate statistical differences: Values with different capital letters (A, B, and C) are significantly different ($p < 0.05$) according to *post-hoc* comparisons.

(± SEM, ranged 48 – 72 h) after the end of treatment. Only females F375 and F381 ovulated, presenting 1 CL each, giving a mean rate of total follicle stimulus of 5.33 ± 1.33 and an ovulation rate of 12.5% (2/16). However, the mean rate of anovulatory follicles was 4.67 ± 1.2. Therefore, no SOV was obtained with this treatment.

For Treatment B, estrous synchronization failed in female F210. Females F261 and F344 displayed behavioral signs of estrus, with subsequent mating with males after CIDR® removal, 53 ± 11 h (± SEM, ranged 42 – 64 h) after the end of treatment. Only female F344 presented 2 CLs, and the total follicle stimulus was 1.33 ± 0.67.

Treatment C was successful in generating SOV response and estrous synchronization in all females. Behavioral signs of estrus were displayed 32 ± 4.62 h (± SEM, ranged 24 – 40 h) after the end of treatment. The mean rate of anovulatory follicles was 2 ± 1.53 and the mean rate of CL was 8 ± 2.52 (± SEM, ranged 3 – 11). This treatment showed a total follicle stimulus of 10 ± 3.46 and an ovulation rate of 80% (24/30) (Figure 4).

To evaluate the effectiveness of superovulation treatments, we analyzed the number of CL, anovulatory follicles (AF), and total follicular stimulus (TFS) across Treatments A, B, and C. The Shapiro-Wilk test indicated that Treatment B data were non-normal ($p < 0.05$), while Treatments A and C followed a normal distribution ($p > 0.05$). Consequently, we applied the Friedman rank test for non-normal data and ANOVA for normal data. The ANOVA revealed a statistically significant difference for Treatment C compared to the other treatments ($p = 0.045$). *Post-hoc* comparisons confirmed a significant difference between Treatments B and C ($p = 0.0332$), while no significant differences were observed between Treatments A and B or A and C ($p > 0.05$).

Embryo recovery was successful in all treatments. However, only one embryo was recovered in Treatments A and B, with the former exhibiting developmental arrest and the latter showing degeneration. As a result, the recovery rate for both treatments was 50% (1/2). In contrast, nine embryos were recovered in Treatment C, which also achieved an ovulation rate of 80% (24/30). Overall, the embryo recovery rate for *B. dichotomus* females was 37.5% (9/24). Of the nine embryos collected, only two were viable (Figure 5). Of the remaining embryos, one was of fair quality, four were degenerated, and two exhibited developmental arrest, following the standards used for bovine embryos (Stringfellow and Seidel, 1999).

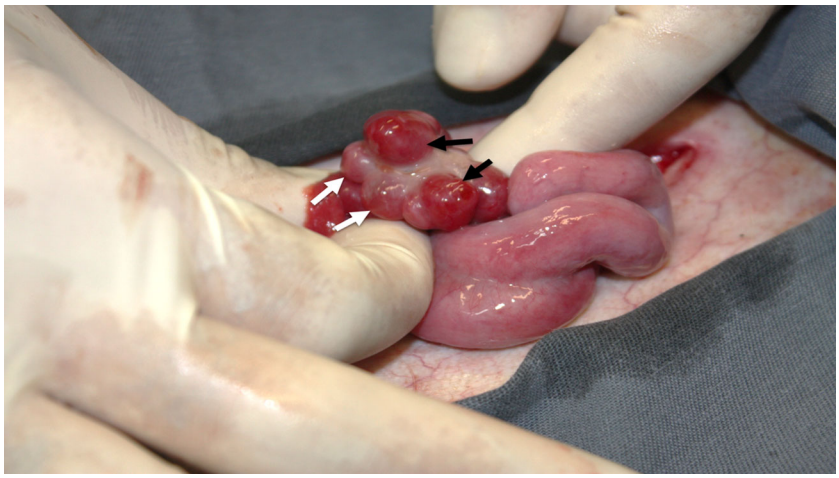

FIGURE 4
Horns, uterine body, and left ovary of the hind F366, of the species *Blastocerus dichotomus* after Treatment C: CIDR® (8 days) + 0.5 mg EB + 0.11 mg GnRH (D0) + 1,200UI eCG (D5) + 530 µl PGF2α (D8) + 2.5 mg LH, 12-18 h after detection of estrus. The presence of anovulatory follicles (white arrows) and corpora lutea (black arrows) are observed.

3.2 Embryo transfer

Before embryo transfer, fawning occurred in October 2015 and the fawn was separated from its mother and transitioned to artificial feeding, subsequently becoming part of the *ex situ* Marsh Deer Conservation Program as a founding female. Then, nine days after the end of synchronization protocol, and upon confirming the presence of the CL through laparoscopic procedure, the uterine horn was exteriorized via median ventral laparotomy (Figure 3B), and two high-quality vitrified embryos were re-warmed and surgically transferred using a "tom cat" catheter.

3.3 Female marsh deer reintroduction into Jataí Ecological Station and monitoring

Once the female was released, monitoring began using a GPS collar with data transmission via GSM on a daily basis (Figure 6). The data collection and transmission schedule functioned perfectly, enabling real-time monitoring of the animal. As expected for open monitoring, the majority (94%) of locations obtained were of high quality. In addition to the planned 9-month monitoring period, tracking continued for an additional 6-month period without any battery issues. During the first three days post-release, the female

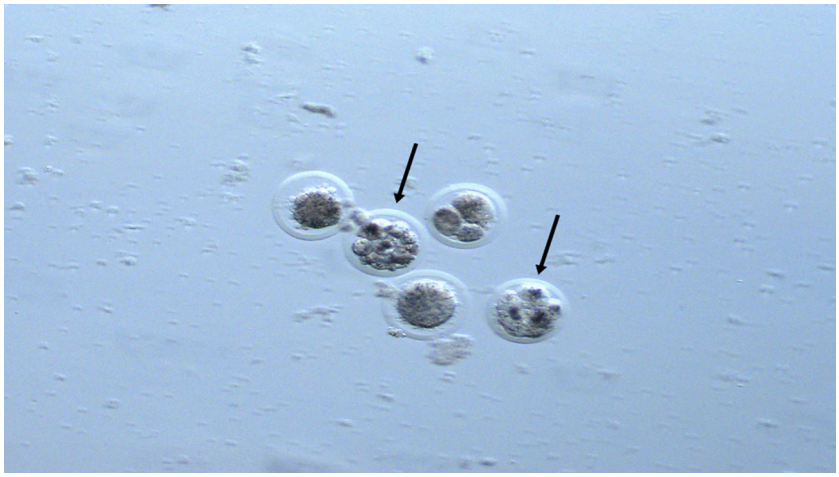

FIGURE 5
Structures recovered from hind F366, of the species *Blastocerus dichotomus* after Treatment C: CIDR® (8 days) + 0.5 mg EB + 0.11 mg GnRH (D0) + 1,200UI eCG (D5) + 530 µl PGF2α (D8) + 2.5 mg LH, 12-18 h after detection of estrus. The black arrows indicate two viable structures for embryo transfer.

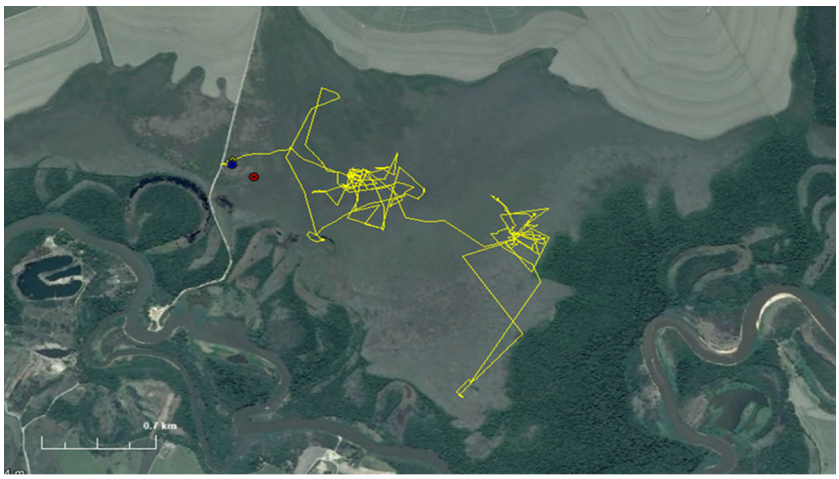

FIGURE 6
Paths taken by the monitored female marsh deer during the first 25 days of return to the wild (yellow), location of the female's capture in July (red), and release in November (blue).

exhibited notably greater movement, averaging 124 meters per hour (approximately 3 km per day). In contrast, movement during the remaining fine-scale sampling (n=4) averaged 50 m/h (approximately 1.2 km/day), with increased movement observed in early January. Throughout the entire monitoring period, the female occupied an area of 2.4 km² (Figure 6).

In August 2016, nine months after female reintroduction into EEJ, a second capture for parturition confirmation was performed. After physical restraint, the female deer underwent an ultrasound to confirm parturition, as female deer typically leave their fawns in secluded areas, hidden in the grass, while they feed to avoid predation. The ultrasonographic evaluation revealed a pregnancy of approximately six months, indicating the loss of the transferred embryos and suggesting natural fertilization three months after reintroduction. After the procedure the female was released at the same point of capture.

4 Discussion

The establishment of the Marsh Deer Conservation Program represents a significant milestone in Brazil and South America's conservation efforts, being one of the first fully local conservation programs. Over the course of two decades, the program has navigated challenges, setbacks, and successes, underscoring the complexities of implementing such a comprehensive initiative with the collaboration of all stakeholders. Despite notable advancements, the marsh deer remains at risk across its range, highlighting the critical importance of integrating reproductive management strategies and biotechnologies to support both at-risk wild and captive populations. While considerable progress has been made in animal reproductive sciences, continued research is essential to furthering our understanding of the reproductive physiology of Neotropical deer species. Based on previous studies of superovulation (SOV) in species such as the brown brocket deer (*Subulo gouazoubira*) (Duarte and Garcia, 1995; Zanetti and Duarte, 2012; Zanetti et al., 2014) and the red brocket deer (*Mazama americana*) (Cursino et al., 2014), this study aims to evaluate the application of SOV and embryo transfer techniques as potential tools for advancing the conservation of the marsh deer.

Due to the limited number of captive marsh deer, we could not use a standard experimental design as used with non-endangered species. Instead, we developed an adaptive system with sequential experiments, making small adjustments between trials based on earlier outcomes. In Treatment A, the inadequate superovulatory response may have resulted from several factors, including handling-induced stress, which affects oocyte maturation, suppress the preovulatory LH surge, and may influence adrenal progesterone production (Monfort et al., 1990; Brann and Mahesh, 1991; González et al., 2008; Zanetti et al., 2014). The absence of an ovulation inducer may have contributed to the lack of ovulation, as the proper timing and type of inducer are critical for follicle maturation and ovulation (Xu et al., 1995; D'Occhio et al., 1997; Liu and Sirois, 1998; D'Occhio et al., 1999; Kaim et al., 2003). Insufficient LH receptor development in ovarian follicles can lead to follicular luteinization without ovulation (Brann and Mahesh, 1991). Common inducers include LH, GnRH and human chorionic gonadotrophin (hCG). Zanetti et al. (2014) observed multiple anovulatory follicles following SOV protocols based on eCG and FSH in brown brocket deer (4.5 ± 1.7 and 7.6 ± 0.9, respectively). Elevated fecal glucocorticoid levels during the SOV period further supported this hypothesis. To address these issues in Treatment B, we added an additional day of exogenous progesterone, adjusted the timing of eCG administration, and included LH as an ovulation inducer after estrus detection.

Modifications made in Treatment B did not effectively induce a superovulatory response in marsh deer females, and female F210 showed no response at all. We suggest that F210's lack of response

may be related to stress from the management procedures during treatment, which could have disrupted her reproductive physiology (Monfort et al., 1990). However, glucocorticoid hormone levels were not measured to confirm this hypothesis. For the other females, we hypothesize that a delayed eCG application may have interfered with the timing of follicular development. By the time eCG was administered, a dominant follicle had already begun to suppress the development of subordinate follicles, which are still FSH-dependent at the recruitment stage (Castilho and Garcia, 2005). Although female F261 developed two ovulatory follicles, there was a lack of synchronicity between follicular maturation and the ovulation inducer. In contrast, female F344 exhibited two CL, which might be considered a superovulatory response, but the overall follicular response was suboptimal due to the limited number of follicles (Table 2).

After the unsatisfactory response to Treatment B, further adjustments were made to the SOV protocol in Treatment C, including lowering the EB dose to 0.5 mg, adding GnRH (0.11 mg), administering eCG on day 5, and delaying LH application until 12-18 hours after estrus detection. These modifications successfully induced an adequate superovulatory response in marsh deer females. Previous studies support the effectiveness of a lower EB dose during progesterone implant insertion (Kumar et al., 2003; Zanetti and Duarte, 2012). Additionally, GnRH improved dominant follicle ovulation, and combining EB with CIDR® promoted follicular atresia in subordinate follicles, aiding estrus synchronization (Bo et al., 1995; Diskin et al., 2002; McCorkell et al., 2008; Zanetti et al., 2014). The delayed eCG application was aimed at optimizing follicular growth, with studies indicating follicular wave emergence occurs 4-5 days after estradiol-17β and progesterone treatment (Burke et al., 2000; McCorkell et al., 2008), and selection occurs 3-4 days after GnRH (Twagiramungu et al., 1995). The delayed LH application allowed for proper follicular maturation, making them more responsive to the LH peak stimulus (D'Occhio et al., 1997, 1999). Despite being 15 years old, female F61 responded to Treatment C, showing 3 CL and 1 anovulatory follicle. Similar to Zebu cattle, older ruminants typically have a reduced ovarian follicle reserve (Peixoto et al., 2006), with a decrease in CL and recovered structures. This suggests that the treatment may be more effective in younger females, as seen with F315 (6 years) and F366 (3 years) (Table 2). The superior response in Treatment C was characterized by higher CL counts and an improved total follicular stimulus (mean CL = 8, TFS = 10) compared to Treatments A and B (mean CL = 0.67 and 1, TFS = 5.33 and 2, respectively). These results suggest that the protocol used in Treatment C significantly enhances follicular development and superovulation outcomes compared to the less effective protocols employed in Treatments A and B.

Embryo recovery was successfully performed in all treatments, with Treatment C yielding the highest recovery rate. The embryo recovery rate in Treatment C was 37.5% (9/24), which is consistent with rates reported in other deer species, such as *Elaphurus davidianus* (29.3%) (Argo et al., 1994); *Cervus elaphus* (43.1%) (Argo et al., 1994), (32.7%) (Asher et al., 1995); and *Dama dama* (30.6%) (Asher et al., 1995). Of the nine embryos recovered in Treatment C, four were degenerated, and two showed developmental arrest. Only two of the remaining embryos were of sufficient quality for transfer and were subsequently used in this study (Figure 5). Both viable embryos came from female F366, who exhibited the best response in terms of follicular development within Treatment C. This suggests that females of optimal reproductive age may respond more favorably to the SOV protocol used in Treatment C, further supporting the importance of age in reproductive success. The results presented here represent an opportunity to adjust the protocol for the implementation of a genetic resource bank for the species. This will be of vital importance for supporting both the captive population and the free-ranging population in the Marsh Deer Conservation Program.

The decision to transfer the only two viable embryos into the same female was a difficult one, made with the intention of maximizing the chances that at least one of them would implant. Although this species is considered uniparous and twin or multiple births have not been observed (Piovezan et al., 2010; Polegato et al., 2018), unfortunately, nine months later, during recapture, it was discovered that the female had lost both embryos. Suggesting that approximately three months after her reintroduction into the wild, it displayed estrus and had the opportunity to mate, eventually showing signs of pregnancy with approximately six months of gestation at the time of the ultrasound evaluation. While this was disappointing in terms of potential genetic diversity enhancement for the EEJ population, it nonetheless provided hope regarding the entire procedure conducted since the female's capture from the wild. It indicates that the surgical procedures for embryo transfer did not negatively affect its physiological reproductive capacity, which is crucial for the viability of this procedure in other wild females.

This attempt demonstrated the feasibility of holding a free-ranging animal in captivity for four months and successfully reintroducing it to the wild, offering valuable insights into the potential of *ex situ* conservation techniques. It is important to note, however, that the female was reintroduced to an area very close (100 meters) to her original capture site, minimizing environmental variations and ensuring she was familiar with the surroundings. The successful reintroduction of this female marsh deer supports findings on the positive effects of longer acclimation periods on site fidelity (Mertes et al., 2019). Consistent with studies on elk (Ryckman et al., 2010; Bleisch et al., 2017), the marsh deer remained near the release site without signs of stress or displacement, suggesting that the extended captivity period facilitated proper acclimation. In contrast, shorter captive holding periods (4–11 days), as seen in elk, led to greater dispersal from the release site (22.6–26 km) (Ryckman et al., 2010). This case highlights the importance of controlled acclimation in familiar environments to improve reintroduction success and reduce risks such as post-release dispersal (Parker et al., 2008; Tuberville et al., 2008). Besides, it is important to consider that deer are prey species in the wild and are highly susceptible to stress stimuli. For this reason, their release must be carefully planned to ensure that the

animal can adequately adapt and return to its natural habitat. Proper planning of reintroduction strategies, including minimizing handling and environmental stressors, is essential for the success of wildlife restoration efforts (Fischer and Lindenmayer, 2000; Seddon et al., 2007). Moreover, stress during reintroduction can negatively impact animal health and survival rates, further highlighting the need for a thorough, well-managed release process (Teixeira et al., 2007).

The use of GPS-GSM technology to monitor the reintroduced female marsh deer provided invaluable insights into her post-release behavior, survival, and adaptation to the wild. Successful reintroduction programs, as highlighted by previous studies, depend on tracking released animals to assess their integration into the environment (Parker et al., 2008; Mertes et al., 2019). In this study, the GPS collar with GSM data transmission was crucial for understanding the female's movements and habitat use post-release. It enabled two types of data sampling: continuous hourly location tracking for broader area uses and survival, and intensive 15-minute intervals to monitor fine-scale movements during the immediate post-release period and some days of gestation. This dual approach provided comprehensive data on both daily behavior and long-term adaptation, aligning with findings from other reintroduction studies where detailed movement data is essential for evaluating habitat selection (Ryckman et al., 2010; Bleisch et al., 2017). Initially, the female showed higher mobility, moving an average of 124 meters per hour (3 km/day) during the first three days, typical of post-release behavior as animals re-establish home ranges (Mertes et al., 2019). Afterward, the female's movement slowed to 50 meters per hour (1.2 km/day), indicating stabilization and adaptation. Therefore, GPS tracking proves a valuable tool for wildlife managers, supporting informed decisions on habitat suitability, release strategies, and the effectiveness of *ex situ* conservation efforts.

This study demonstrates the potential of embryo transfer as a viable strategy for enhancing genetic diversity in natural marsh deer populations, particularly those threatened by low genetic variability (Rola et al., 2021, 2023). Traditional reintroduction programs often face challenges related to inbreeding depression, which can lead to reduced fitness, lower reproductive success, and heightened disease susceptibility (Seddon et al., 2007). By introducing genetically diverse embryos sourced from *ex situ* conservation programs, this technique offers a controlled and scientifically grounded approach to mitigating these issues. The findings highlight key advancements, including improvements in hormonal protocols, optimized embryo collection techniques from the captive population, and the refinement of transfer methods. The success achieved in obtaining viable embryos and performing transfers indicates that these methods can significantly contribute to conservation programs to sustain healthy and diverse populations. Continued research and technological refinement are necessary to enhance the effectiveness of this approach and adapt it to the specific reproductive physiology of marsh deer and other threatened Neotropical deer species. By integrating embryo transfer into broader conservation strategies, this method holds promise as a valuable tool for addressing genetic bottlenecks and improving the long-term viability of wildlife populations facing environmental and genetic challenges.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Ethics Committee on Animal Use (CEUA). The study was conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any identifiable images or data included in this article.

Author contributions

DG: Conceptualization, Investigation, Visualization, Writing – original draft. PP: Conceptualization, Investigation, Visualization, Writing – review & editing. EZ: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. MO: Conceptualization, Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing. LR: Conceptualization, Investigation, Visualization, Writing – review & editing. JD: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft.

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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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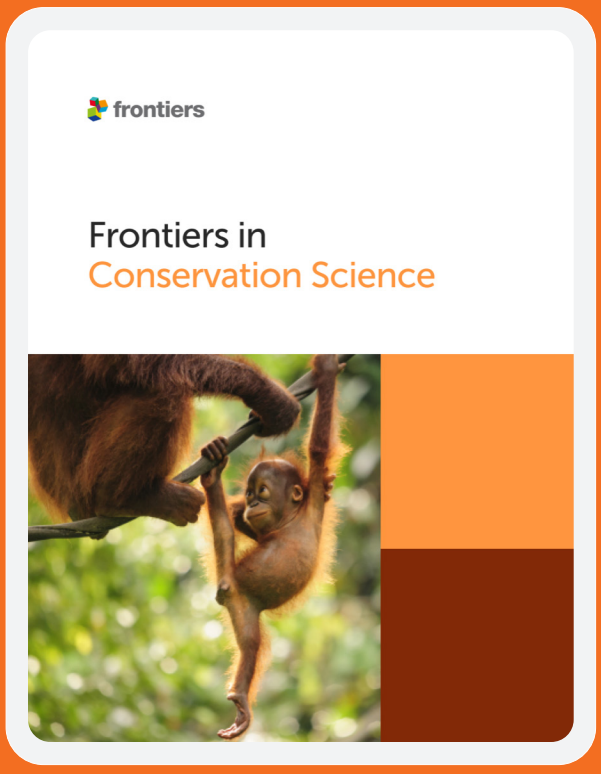

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