INDIVIDUAL VARIATION AND RESPONSES OF ANIMALS TO CHANGING ENVIRONMENTS

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INDIVIDUAL VARIATION AND RESPONSES OF ANIMALS TO CHANGING ENVIRONMENTS

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Individual Variation in Dietary Wariness Is Predicted by Head Color in a Specialist Feeder, the Gouldian Finch

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Shifts in resource availability due to environmental change are increasingly confronting animals with unfamiliar food types. Species that can rapidly accept new food types may be better adapted to ecological change. Intuitively, dietary generalists are expected to accept new food types when resources change, while dietary specialists would be more averse to adopting novel food. However, most studies investigating changes in dietary breadth focus on generalist species and do not delve into potential individual predictors of dietary wariness and the social factors modulating these responses. We investigated dietary wariness in the Gouldian finch, a dietary specialist, that is expected to avoid novel food. This species occurs in two main head colors (red, black), which signal personality in other contexts. We measured their initial neophobic responses (approach attempts before first feed and latency to first feed) and willingness to incorporate novel food into their diet (frequency of feeding on novel food after first feed). Birds were tested in same-sex pairs in same and different head color pairings balanced across experiments 1 and 2. Familiar and novel food (familiar food dyed) were presented simultaneously across 5 days for 3 h, each. Gouldian finches fed on the familiar food first demonstrating food neophobia, and these latencies were repeatable. Birds made more approach attempts before feeding on novel than familiar food, particularly red-headed birds in experiment 1 and when partnered with a black-headed bird. Individuals consistently differed in their rate of incorporation of novel food, with clear differences between head colors; red-headed birds increased their feeding visits to novel food across experimentation equaling their familiar food intake by day five, while black-headed birds continually favored familiar food. Results suggest consistent among individual differences in response to novel food with red-headed birds being adventurous consumers and black-headed birds dietary conservatives. The differences in food acceptance aligned with responses to novel environments on the individual level

(found in an earlier study) providing individuals with an adaptive combination of novelty responses across contexts in line with potential differences in movement patterns. Taken together, these novelty responses could aid in population persistence when faced with environmental changes.

Keywords: food neophobia, dietary conservatism, *Erythrura gouldiae,* color polymorphism, novelty syndrome, specialist, conservation

INTRODUCTION

Human activities are increasingly confronting animal species with environmental challenges such as changes in habitat, which affects resource availability. New food resources may appear, such as the emergence of invasive species or incidental food provisioning by humans, meanwhile preferred food may disappear as native habitats dwindle. The ability to adapt to changes in food resources, has far-reaching consequences on distribution and population development (McKinney, 1997). However, a species' response to novelty is rarely uniform, but harbors considerable among individual variation (personality) in response to environmental challenges (White et al., 2013; Boulton et al., 2014; Edwards et al., 2016). Additionally, seemingly independent behaviors can be correlated, forming behavioral syndromes that define an individual's response across contexts (e.g., Sih et al., 2015). Such among individual variation reflects different strategies to cope with environmental stressors, giving certain individuals advantages depending on environmental conditions (e.g., Dingemanse et al., 2004). Therefore, it has been proposed that populations with individual differences improve a species' ability to respond to environmental change (Delarue et al., 2015). Accordingly, understanding the overall level and individual differences within a species' response to novel resources may be of conservation value (Kelleher et al., 2018).

Responses to novel food consist of two different and independent processes. First animals must overcome neophobic responses toward the sight or smell of novel food. Then they must incorporate the novel food consistently into their diet, i.e., dietary conservatism (Marples and Mappes, 2011). Both processes together are termed dietary wariness (Marples and Kelly, 1999; Kelly and Marples, 2004; Marples et al., 2007; McMahon et al., 2014). Food neophobia is an adaptive mechanism to avoid potentially harmful substances and has been shown to be a widespread behavioral strategy demonstrated in humans (Cooke et al., 2007; Knaapila et al., 2007), non-human primates (Johnson, 2000; Visalberghi and Addessi, 2000; Visalberghi et al., 2003; Gustafsson et al., 2014; Forss et al., 2019), rodents (Hall et al., 1997; Lin et al., 2009; Modlinska et al., 2015), carnivores (Malmkvist et al., 2003), and birds (Marples et al., 1998; Camín et al., 2016). It has a genetic component (Jones, 1986; Marples and Brakefield, 1995; Turro-Vincent et al., 1995; Boliver and Flaherty, 2004; Cooke et al., 2007; Knaapila et al., 2007), though social and asocial environmental factors and experience modulate food neophobia (Marples et al., 2007; Doktorovová et al., 2019). For example, naïve individuals are more likely to try novel food that they have seen others consume (Dally et al., 2008; Chiarati et al., 2012; Greggor et al., 2016b). Moreover, group composition (Oostindjer et al., 2011) and an individual's own position in a group (Amici et al., 2020) can affect food neophobic responses. Finally, juveniles have been found less food neophobic than adults in some primates (Visalberghi et al., 2003; Ueno and Matsuzawa, 2005; Addessi and Visalberghi, 2006; but see Gustafsson et al., 2014; Arnaud et al., 2017). While studies generally find considerable differences in food neophobia between individuals (e.g., Marples et al., 1998; Exnerova et al., 2010; Liebl and Martin, 2014), few studies have investigated whether or not individuals are consistent in these differences. Coleman and Wilson (1998) demonstrated consistent individual differences in food neophobia in fish, as did Prasher et al. (2019) in birds, indicating that food neophobia forms part of personality traits.

Fewer studies have looked into the neophilic or exploratory component of sampling novel food, which prompts an individual to approach and collect information about the unfamiliar food source. Chimpanzees (*Pan troglodytes*) – who are food neophobic – have been found to extensively explore novel food items before first tasting, and also rely heavily on social information (Gustafsson et al., 2014). Likewise, mink (*Mustela vision*) sniffed more often and for longer on novel than familiar food (Malmkvist et al., 2003). Forss et al. (2019) found that ape species with a more solitary lifestyle relied more on individual exploration of novel food than more social ape species who used social observation. While exploration of novel objects or environments has been shown to be consistent individual traits (Williams et al., 2012; Boulton et al., 2014), there are no studies that have tested this for novel food.

Once novel food has been tasted, food neophobia terminates and dietary conservatism begins. Dietary conservatism describes the process of incorporating novel food into the diet over time (Marples et al., 1998; Marples and Kelly, 1999; Kelly and Marples, 2004), and can be divided into two stages; an assessment stage where novel food is occasionally sampled, but not preferred, and a full acceptance stage in which novel food is consumed at equal or higher rates than familiar food (Marples and Kelly, 1999). Dietary conservatism is often assessed by comparing the amount of novel versus familiar food consumed, with novel food often ingested at a lower rate than familiar food (Malmkvist et al., 2003; Visalberghi et al., 2003; Gustafsson et al., 2014). Individuals fall into two genetically different types – adventurous consumers and dietary conservatives (Marples and Brakefield, 1995; Marples et al., 1998, 2007; Thomas et al., 2003, 2004). Adventurous consumers accept the novel food as soon as neophobia has ceased, whereas dietary conservatives demonstrate a prolonged aversion (sometimes months or years) to accept novel food into

the diet (Marples and Brakefield, 1995). Both types are found on the species level in quails and a wide range of passerines, with dietary conservative individuals comprising between 30–50% of many populations (Thomas et al., 2010). The two foraging strategies may reflect different risk-reward trade-offs (Toscano et al., 2016), with adventurous consumers maximizing food intake through a generalist foraging approach at the risk of occasional food poisoning, while dietary conservatives (e.g., specialist foragers) may have high efficiency in exploiting a few familiar resources without risk (Thomas et al., 2010). Whether adventurous consumers and dietary conservatives also reflect consistent individual strategies is unclear as some studies have found consistency (Thomas et al., 2010), whereas others did not (Marples et al., 1998; Prasher et al., 2019).

Most studies on dietary wariness focus on the phenomenon itself and its mechanisms. Few studies have investigated how environmental conditions affect dietary wariness or how dietary wariness is linked to other traits. Two general hypotheses should be mentioned. The Neophobia Threshold hypothesis predicts that neophobia preserves ecological specialization (Greenberg, 1983) and limits ecological plasticity, having been confirmed in closely related diet and habitat specialist and generalist species, with specialists showing more (spatial) neophobia when encountering novel micro-habitats (Greenberg, 1983, 1984). The highly specialized snail kite (*Rostrhamus sociabilis*) serves as a supporting example of a species with both high food neophobia and diet specialization, since they reject even similar but unfamiliar snails (Beissinger et al., 1994). The Dangerous Niche hypothesis, in contrast, addresses the general risk inherent to the environment rather than ecological plasticity (Greenberg, 2003). In support of this hypothesis, higher neophobia has been demonstrated in species that are more likely to encounter dangerous situations, such as poisonous prey items (Greenberg, 2003; Mettke-Hofmann et al., 2013). Camín et al. (2016) compared dietary wariness between the rufous collared sparrow (*Zonotrichia capensis*), a dietary specialist, and the many colored chaco finch (*Saltatricula multicolour*), a dietary generalist. While both species had similar latencies to feed on the novel food, the generalist took significantly longer to taste the novel than the familiar food showing clear food neophobia. Interestingly, the proportion of dietary conservative individuals in the generalist species was 37%, whereas it was only 12% in the specialist species. The authors concluded that the generalist encountered more food containing toxic secondary compounds causing food neophobia, a result which was consistent with the Dangerous Niche hypothesis (Greenberg, 2003) but in contrast to the Neophobia Threshold hypothesis (Greenberg, 1983). This is one of very few studies involving specialists as most studies focus on generalist foragers (Turro-Vincent et al., 1995; Marples et al., 1998; Kelly and Marples, 2004).

Responses toward novel food may have ramifications beyond diet. A newly emerging area of research connects movement and dietary wariness since animals moving into unfamiliar environments are also more likely to encounter novel food. For example, in invasive species lower food neophobia was found in populations at the invasion front as compared to native or more established populations in two bird species (Martin and Fitzgerald, 2005; Cohen et al., 2020). Lower food neophobia helps to adapt to unfamiliar environments (Martin and Fitzgerald, 2005; Cohen et al., 2020). Martin and Fitzgerald (2005) also measured the amount of novel food consumed, which did not differ between the established and invading populations of house sparrows (*Passer domesticus*). While this study showed house sparrows from native and invasive populations consumed similar amounts of novel food, they found the invader was less neophobic. In other scenarios, lower food neophobia could theoretically give invading species a competitive edge over native ones, thereby compromising the persistence of native species. With changes in climate and species distribution shifts documented globally (Perry et al., 2005; Boisvert-Marsh et al., 2014; Pacifici et al., 2017; Cohen et al., 2019), species that are likely most at risk of population declines are those that occupy a specialist lifestyle. Therefore, investigating dietary wariness in specialized species could help predict certain vulnerability to changes in resources or competitors.

Due to the potential importance of dietary wariness for long-term population persistence, and the lack of knowledge how specialist species deal with novel food, we studied dietary wariness in the Gouldian finch (*Erythrura gouldiae*), a diet and habitat specialist. The Gouldian finch predominantly forages on grass seeds, particularly annual Sorghum species (Brazill-Boast et al., 2011). Changes in food availability caused by grazing and changed fire regimes have resulted in steep population declines (Dostine et al., 2001; Legge et al., 2015; Weier et al., 2016, 2017, 2018), with the species now listed as endangered by the Australia Government (Environment Protection and Biodiversity Conservation, EPBC, 2018). Higher stress levels and lower physiological condition scores in response to food shortages, particularly during the wet season, have been reported in this food specialized species compared to other sympatric but more generalist finches (Maute et al., 2013).

Gouldian finches are a unique example of a non-melanin-based color-polymorphism, where head colors co-exist in sympatry in the wild with 70% black-headed, 30% red-headed and <1% yellow-headed birds (Kim et al., 2019). *In situ* research has shown head color signals personality; black-headed birds are consistently less aggressive yet readily investigate changes in their familiar environment (novel objects) and take greater risk in potentially dangerous situations than red-headed birds (Williams et al., 2012). However, black-headed birds hesitate longer to enter unsuitable novel habitats (Mettke-Hofmann et al., 2020). The combination of high interest in changes in the familiar environment and less interest in entering unfamiliar environments in the black-headed birds is consistent with a resident cognitive strategy (Mettke-Hofmann et al., 2020). This allows tracking of changes in the familiar environment facilitating persistence at a site (Mettke-Hofmann et al., 2005, 2012). In contrast, the higher willingness of red-headed birds to enter unfamiliar environments but refraining from investigating changes in the familiar environment is consistent with a migratory/nomadic cognitive strategy (Mettke-Hofmann et al., 2020). Moreover, morph composition has been shown to affect novelty responses in Gouldian finch social groups. The presence of black-headed birds increased cautious behavior toward novel

environments in other Gouldian finches, whereas red-headed birds did not (Mettke-Hofmann et al., 2020).

Besides their extreme food specialization, little is currently known about Gouldian finches' responses (a) to unfamiliar food and their willingness to incorporate novel food into the diet, (b) whether head colors and/or personality types respond differently to novel food and (c) how morph group composition may affect responses. However, such responses could have far-reaching consequences for population recovery in light of further habitat change. Moreover, few food neophobia studies have been conducted on food specialists and color polymorphism has only been considered from a predator perspective, i.e., how rare color morphs can induce neophobic reactions and dietary conservatism in predators (Thomas et al., 2003, 2010). The current study aimed to investigate the entire process of dietary wariness in the food specialized Gouldian finch, considering potential differences in responses of color morphs reflecting underlying differences in personality, and in relation to group composition. We combined two experimental approaches for studying dietary wariness: (1) we investigated food neophobia by considering both, food neophobia and food neophilia as separate processes consistent with other studies (e.g., Gustafsson et al., 2014; Forss et al., 2019). (2) We investigated the process of dietary conservatism following the approach by Marples et al. (1998) which distinguishes food neophobia from dietary conservatism. To avoid confusion between the umbrella term of dietary conservatism describing the process of accepting novel food and one of its outcomes (i.e., being dietarily conservative) we will refer to the process as the rate of incorporation of novel food into the diet.

Based on the existing literature and the dietary specialism of Gouldian finches, the following predictions were made.

Prediction 1 – Individual consistency: In line with other studies, we expected consistent among individual variation in food neophobia (Coleman and Wilson, 1998; Prasher et al., 2019). This may extend to the willingness to incorporate novel food into the diet.

Prediction 2 – Effects of color morphs/personalities: Morphs have been found to reflect different personalities (Mafli et al., 2011; Mateos-Gonzalez and Senar, 2012; Williams et al., 2012). In the Gouldian finch, black-headed birds' personalities combine into a resident cognitive strategy, whereas in red-headed birds they align with a migratory/nomadic cognitive strategy (Mettke-Hofmann et al., 2020). We therefore expected black-headed birds to be more food neophobic than red-headed birds. The more spatially novelty-prone red-headed birds are likely to encounter unfamiliar food on a regular basis and sampling novel food may be part of their cognitive adaptation (Martin and Fitzgerald, 2005; Cohen et al., 2020). Whether this extends to dietary conservatism is unclear (Martin and Fitzgerald, 2005) but if so, we expect red-headed birds to incorporate novel food faster than black-headed birds facilitating their higher movement potential.

Prediction 3 – Evidence for a novelty syndrome: As the Mettke-Hofmann et al. (2020) study tested exactly the same birds in the same setting as in the current study, we were able to test for a novelty syndrome. We expected a positive correlation between spatial and food neophobia on the individual level, which would equip birds with a high propensity to enter novel environments with a high willingness to try and accept novel food.

Prediction 4 – Social effects: Group composition can affect foraging efficiency (Paijmans et al., 2020) and responses to novel food (Oostindjer et al., 2011). Different scenarios are possible: (a) If red-headed birds are less food neophobic than black-headed birds (see prediction 2), then pure red-headed pairs may be fastest to sample and incorporate novel food into their diet, whereas pure black-headed pairs may be the most food neophobic. Mixed pairs may fall in between, with either black-headed birds slowing down red-headed birds, similar to their influence on spatial neophobia (Mettke-Hofmann et al., 2020), or red-headed birds reducing neophobia in black-headed birds. (b) Alternatively, mixed morph pairs may be fastest as studies have found higher foraging efficiency and faster approach to novel feeders in mixed personality groups as compared to groups consisting of one type only (Dyer et al., 2009; Paijmans et al., 2020).

Prediction 5 – Dietary wariness: We expected to find species-level dietary wariness, in line with the neophobia threshold hypothesis (Greenberg, 1983). Gouldian finches are food specialists feeding nearly exclusively on Poaceae, e.g., Sorghum spec (Dostine and Franklin, 2002), a plant group that has very low toxicity levels (Diaz, 1996). Therefore, we predicted (a) food neophobia evidenced by hesitating longer before feeding on novel food in comparison to familiar food, and (b) dietary conservatism evidenced by birds continuing to prefer familiar over novel food after any initial neophobia has ceased.

MATERIALS AND METHODS

Study Group and Housing

Thirty-two Gouldian finches originating from 12 private breeders were used. Birds were acquired at roughly 1 year old and had spent different amounts of time in our Animal Facility, but at least 2 months before the experiments began. All birds were wild type, parent reared, and ages ranged from 1 to 6 years. Sex ratios were equal with 16 males (eight red-head, eight black-head) and 16 females (seven red-head, nine black-head). All birds were housed together within six free-flight cages (1.20 m long × 80 cm deep × 1.00 m high) in groups of 5–6 individuals. All birds were grouped in mixed sexes, ages and head colors with the exception of the 1-year-old individuals (10 birds) who were housed in same sex groups. Birds were fed a 6:3:1 mixture of 6 units Astrilden Spezial, 3 units Amadinen-Zucht Spezial and 1 unit red sibirica millet (referred to as familiar seed hereon), plus grit (all purchased from Blattner-Heimtierfutter, Ermengerst, Germany) and egg shells in separate feeders located at the front of the cage. French red spray millet (Blattner-Heimtierfutter) was located next to the feeders. Water was available *ad libitum*. Once per week Blattner's vitamins (Blattner-Hiemtierfutter) were supplemented in the drinking water. Birds were kept at a temperature of 24°C and 51% humidity and provided with a full spectrum light source with a light:dark cycle of 13:11 h. In addition to the two wooden perches located within each cage, natural branches and twigs were available.

Experimental Set Up

Testing took place in four experimental cages (1.20 m long × 0.7 m deep × 1.00 m high) in a separate room from the housing. Experimental cages each comprised of three wooden walls and a wire mesh front and ceiling. Each cage was furnished with a front perch, running parallel to the front wire mesh with two plastic plant pot saucers (14 cm diameter × 2.50 cm depth) as feeders attached side-by-side between the mesh and the perch (**Figure 1**). Two additional perches at the same height as the first perch were positioned on the left- and right-hand side of the cage running perpendicular to the front perch with a water dispenser each attached to it from the outside. The front perch was marked at 7.5 cm away from each feeder as this is the average body length of a Gouldian finch and was used to determine distance within the data collection sessions. Birds in different cages could not see each other but were in auditory contact. A digital video camera was positioned on a tripod one meter in front of each experimental cage, connected to GeoVision 1480 recording software for later analysis.

In the experimental cages, birds were fed two types of food. Familiar food was the seed they were fed in their normal housing. Novel food was produced by dying the familiar food, which is a common procedure to create novelty (e.g., Marples et al., 2007; Greggor et al., 2016b). Novel seed were either green (peppermint green pastel paste gel edible concentrated food coloring) for experiment 1 or red (scarlet red pastel paste gel edible concentrated food coloring; both from Sugarflair Colours Limited, Essex, United Kingdom) for experiment 2 (**Figure 1**). We selected glycerine-based products over sugar-based ones to minimize effects of taste. The seed was dyed by boiling 50 parts seed (g) in a solution of 1 part dye (ml) with 100 parts water (ml) for 20 min at a low heat. Dyed seeds were dried at room temperature for 24 h then separated into 20 g portions and frozen at a temperature of –21°C until required at which point it was defrosted early morning prior to use. Gouldian finches readily consume undyed boiled seeds as they may be more palatable than dry seeds (CMH, own obs.).

Procedure

Birds were assigned to same sex pairs for testing as Gouldian finches are highly social and testing in isolation would produce unnatural conditions (Brush and Seifried, 1968; Mettke-Hofmann, 2012). It also allowed testing for effects of group composition. Same sex pairs were matched for size and weight. Age was controlled for by pairing birds that were at least 2 years apart as age effects have been shown to influence object neophobia in this species (Mettke-Hofmann, 2012). One bird in each pair was fitted with two white leg bands (one per leg) for identification purposes and all birds were weighted and their tarsus length taken as a measure of body size. To investigate effects of group composition, each bird was tested once in a same head color pair (red-red or black-black) and once in a different head color pair (red-black) in two separate experiments. In experiment 1, half of the birds were paired with a partner of the same head color and half of the birds with a partner of the opposite head color. In experiment 2, which took place after all birds had finished experiment 1, the pairings were reversed so that birds who were paired with a partner of the same head color in experiment 1 were paired with a bird with the opposite head color in experiment 2 and vice versa. As we had more black-headed than red-headed females and uneven numbers within head colors, two black-headed females were tested with partner birds in both experiments that had gone through their own testing already (hereafter named experienced partner bird). In experiment 2, two additional black-headed females and one red-headed female

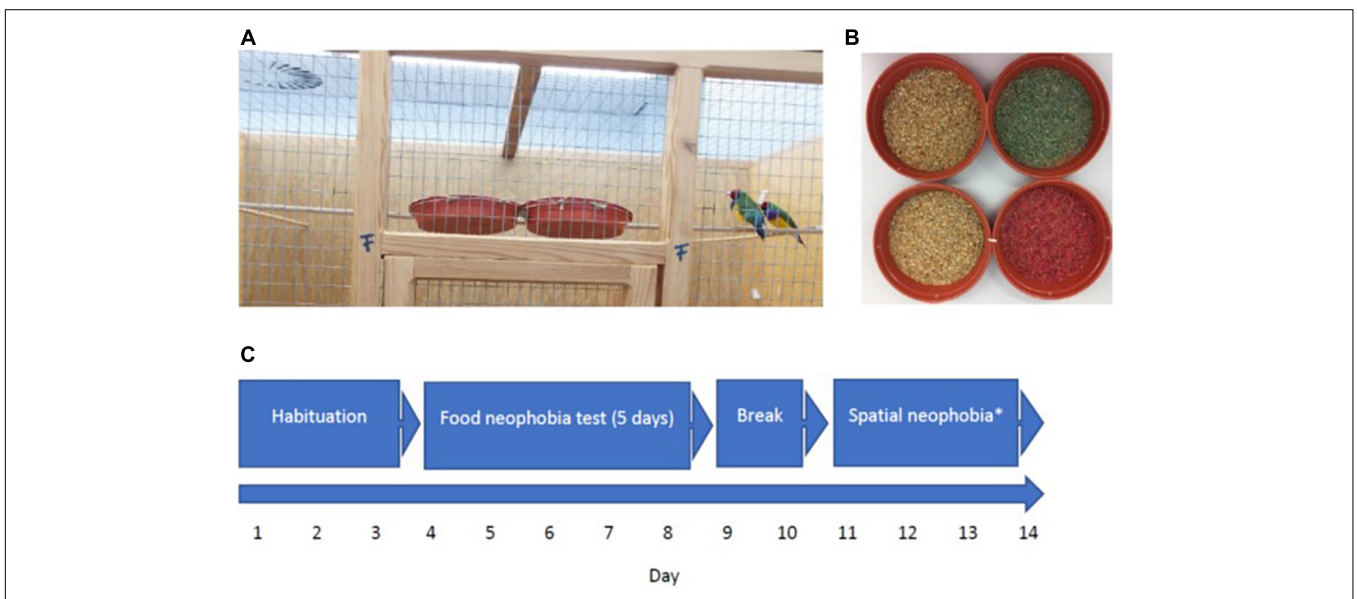

FIGURE 1 | (A) Upper part of experimental cage with the two feeders side-by-side, **(B)** familiar (left) and novel green (experiment 1) and red (experiment 2) food (right), **(C)** flow chart demonstrating experimental design (repetition of experiment not shown); *see Mettke-Hofmann et al. (2020).

were tested with experienced partner birds. Only the data from the focal birds were included from these pairings. The interval between experiment 1 and 2 for individual birds ranged from 3 to 18 weeks.

Four pairs were tested simultaneously with a total of four batches of four pairs, each. Head color combination and age were balanced across cages and batches. Birds could settle and feed on their standard (familiar) food in both feeders in the experimental cages for 3 days prior to the start of testing. Experiments ran from day 4 to day 8 followed by spatial neophobia testing between day 11 and 14 (Mettke-Hofmann et al., 2020) after which birds were moved back into their home cage (**Figure 1**).

During experiment 1, each morning for five consecutive days the two feeders containing the familiar food were removed for 1 h (8:00 to 9:00 AM) directly after the lights went on to control for hunger levels at the start of testing. At 9:00 AM each day the feeders were returned, but this time only one contained the familiar seed, whereas the other one contained the novel green seed. The birds' feeding behavior was video recorded for 3.5 h. At the end of each session the novel food was removed from the experimental cage and replaced with familiar food. The positions of the familiar and novel food were counter-balanced to the left and right locations across days within and across cages. Once all birds had completed experiment 1, they went through experiment 2 following the same procedure as before except birds were paired with a new partner in a different head color combination than in experiment 1 and the novel seed was red to retain novelty. Due to the new pairing, it was not possible to counter-balance novel food colors within experiments.

Data Preparation

Data preparation and statistical analyses were performed in R version 3.6.0. (R Core Team, 2017). Raw data can be found in the **Supplementary Materials S1,S2**. To assess neophobia we extracted two response variables: approach frequency prior to first feed and latency to first feed. Approach frequency before feeding reflects the conflict between the motivation to approach and feed (food neophilia) and the motivation to avoid the unfamiliar food due to its potential harmfulness (food neophobia) and helps identifying the contribution of each motivation to the latency to feed (Mettke-Hofmann et al., 2009). Meanwhile, latency to feed is a standard measure of neophobia (e.g., Camín et al., 2016; Forss et al., 2019). Response variables were not correlated with body mass (Spearman correlation: n = 31, familiar food: approach frequency, corr coef = 0.05, P = 0.791, first feeding latency, corr coef = 0.306, P = 0.96; novel food: approach frequency, corr coef = 0.135, P = 0.469, first feeding latency, corr coef = 0.159, P = 0.392) or body size (familiar food: approach frequency, corr coef = –0.013, P = 0.946, first feeding latency, corr coef = 0.177, P = 0.341; novel food: approach frequency, corr coef = 0.128, P = 0.491, first feeding latency, corr coef = 0.022, P = 0.907).

To assess dietary conservatism, the rate of incorporation of novel food into the diet was measured as feeding frequency following the first feed on each food type. Feeding frequency was recorded for each of the 5 days of testing in experiment 1 and experiment 2, separately.

All three response variables did not meet the requirement of normality in raw or transformed form. Therefore, untransformed data were used, and appropriate model error structures specified. Sample size was N = 31 birds for the analysis of neophobia, as one bird died between experiment 1 and 2 due to circumstances unrelated to the experiments and their data were therefore removed from the study. Sample size for assessment of dietary conservatism was N = 30 birds, due to a transcription error resulting in missing data for one bird for frequency of feeding after the first feed.

Statistical Analysis

To address prediction 1 about consistent among individual variation, we assessed repeatability (R) of behavior by accounting for the degree of variation attributable to bird identity using the rptR package (Stoffel et al., 2017). Repeatability highlights persistent differences in novelty reactions between individuals (Dingemanse et al., 2003; Nakagawa and Schielzeth, 2010). All birds had also been tested on spatial neophobia directly following the food neophobia testing (see **Figure 1**; Mettke-Hofmann et al., 2020), allowing us to test for a novelty syndrome (prediction 3). From their familiar test cage, birds gained access on 1 day to a novel open habitat and on another day to a novel dense habitat. Approach frequencies before entering and latencies to enter the novel habitats were measured. Like in the current study, all birds were tested in same and mixed head color combinations across the two experiments. We correlated the variables for spatial neophobia with the dietary wariness measures (approach frequency and latency to first feed for novel and familiar food, frequency of feeding visits after first feed to novel and familiar food on day 1 and day 5) of experiment 1 only using a Spearman rank correlation. Responses in the first experiment were chosen to exclude any habituation effects.

To address predictions 2 (effects of color morphs/personalities), 4 (social effects) and 5 (species-level dietary wariness), we fitted generalized linear mixed models (GLMM) for all three response variables using the R package 'lme4' version 1.1-20 (Bates et al., 2015), with a specified Poisson family error distribution with log-link function. We developed four statistical models tailored to our predictions. One model to address predictions 2 and 5 for food neophobia (model A) and a second model to address prediction 4 about social effects of morph composition within pairings on food neophobia (model B). Note that we had two response variables for model A and B. Similarly, we had one model to address prediction 2 and 5 for dietary conservatism (model C) and another one to address prediction 4 for dietary conservatism (model D). The analyses were conducted as separate models to avoid inclusion of too many variables in any single model which would cause over-parametrization issues (Crawley, 2012). Bird identity, partner identity and cage number were entered as crossed random effects in all models (crossed rather than nested because assigning birds to new pairings for experiment 2 precluded birds being tested in the same cage as in experiment 1). To account for using the same data in both models (for food type and head color) we used sequential Bonferroni adjustments were necessary (Rice, 1989; Chandler, 1995).

Generalized Linear Mixed Models for Food Neophobia

Model A: Approach to and Feed on Novel Food

Response variables were approach frequency prior to first feed and latency to first feed. Each GLMM contained two predictor variables: food type (familiar, novel) and head color morph (black, red); with two control variables: age [1 year old (N = 10), older than 1 year (N = 21)]; and experiment (1, 2; to account for the repeated testing). Sex was not included as earlier screening showed no effect of sex which corroborates our previous findings in other contexts that sex did not influence neophobic responses (Eccles, 2018; Mettke-Hofmann et al., 2020). Food type, morph and experiment were entered as a three-way interaction term in the full model as morphs were expected to differ in their response to novel food and these differences may be more prevalent during the first experiment. Sample sizes in the three-way interaction for all comparisons were N = 31 birds (124 rows of data) as all birds were tested in both experiments with both food types. Where the three-way interaction was not significant, we tested the following two-way interactions: food type × morph, food type × experiment and morph × experiment.

Model B: Social Factors Influencing Neophobia

Response variables were approach frequency prior to first feed and latency to first feed. Each GLMM contained three predictor variables: food type (familiar, novel), head color morph (black, red) and partner head color (black, red) and one control variable: relative age within each pairing (younger or older to account for age effects within pairings as found in earlier studies; Mettke-Hofmann, 2012). We included food type, morph and partner head color as a three-way interaction term as the combination of morphs may influence behaviors (e.g., Dyer et al., 2009). Where the three-way interaction was not significant, we tested the following two-way interactions: food type × morph, food type × partner head color and morph × partner head color.

Generalized Linear Mixed Models for Dietary Conservatism

Model C: The Rate of Incorporation of Novel Food Into the Diet

The response variable was the feeding frequency on each food type after first feed. The GLMM contained three predictor variables: food type (familiar, novel), morph (black, red) and day (1 – 5); and three control variables: age (1 year old, older than 1 year), experiment (1, 2) and latency to feed (a continuous variable to control for the variation in time that feed frequency was recorded for each bird and which was scaled to a mean of 0 and SD = 1 to aid model interpretation). Food type, morph, and experiment were entered as a three-way interaction term, as were food type, morph and day. Sample sizes in the three-way interaction for all comparisons were N = 30 birds (120 rows of data) as all birds were tested in both experiments with both food types. Where the three-way interactions were not significant the following two-way interactions were tested: food type × morph, food type × experiment, morph × experiment, food type × day, morph × day and experiment × day.

Model D: Social Factors Influencing Dietary Conservatism

The response variable was the feeding frequency on each food type after first feed. The model contained three predictor variables: food type (familiar, novel), morph (black, red) and partner head color (black, red) and two control variables: relative age within each pairing and latency to first feed. Food type, morph, and partner head color were included as a three-way interaction term, with subsequent two-way interactions: food type × morph, food type × partner head color and morph × partner head color in case of a non-significant outcome.

Model Simplification for Generalized Linear Mixed Models

Interaction terms were retained where $P < 0.05$, and excluded where they failed to reach this criterion, in a stepwise model simplification, following Crawley (2012). Orthogonal data are robust to stepwise removal of interaction terms as variation attributable to each factor is constant at each stage of the stepwise simplification (Crawley, 2007). All predictor and control variables were retained in all final models. Retaining fixed effects in final models minimized repeated testing and hence concern about the risk of type I errors (e.g., Steel et al., 2013) and increased our ability to interpret model output and effect size calculations in a biologically meaningful way (e.g., Nakagawa and Cuthill, 2007). We adjusted convergence tolerance using the arguments 'allFit' and 'control' to specify the optimizer to 'bobyqa' and increased the number of iterations to 100,000, a practice considered 'gold standard' for ensuring stable model fit (Bates et al., 2019). Model fit was assessed by visually inspecting plots of fitted model residuals to ensure an even spread of residuals, which we found in all cases. We assessed each final model by comparing it against the null model (an identical model except for the removal of the predictor and control variables, with an intercept of 1 specified) using the anova command in R. The final model was only accepted where it was a significantly better fit than the null model (Burnham and Anderson, 2002).

We checked for evidence of collinearity within models using the function 'vif' (variance inflation factor) in the package 'car,' and extracted effect sizes using the r.squaredGLMM command in the package MuMIn (Barton, 2015). To facilitate future meta-analyses, we report both marginal and conditional effect sizes, r^2m and r^2c respectively, where r^2m explains variance due to fixed effects and r^2c explains variance due to fixed and random effects (Nakagawa and Cuthill, 2007; Nakagawa and Schielzeth, 2013).

Ethical Note

We conducted all experiments in accordance with published guidelines for the treatment of animals in behavioral research (ASAB/ABS guidelines, Animal Behaviour, 2018; ARRIVE guidelines, Kilkenny et al., 2010). Holding and experimental aviaries conformed to Home Office codes of practice (Home Office, 2013) and were carried out in approved facilities within Liverpool John Moores University. All experiments were non-regulated by the Home Office and complied with the ethical and

welfare guidelines for animals and the legal requirements of the University (CMH_GE/2016-5) and the United Kingdom.

RESULTS

Food Neophobia

All birds fed on the familiar food on the first day of presentation in both, experiment 1 and experiment 2. There was variation between birds in day of first feed on the novel food. In experiment 1, 20 birds fed on the novel food (green seed) on the first day, one bird on the second day, four birds on the third day and two birds on the fourth day. Four birds never fed on the novel food in experiment 1. In experiment 2, all birds fed on the novel food (red seed): 20 birds on the first day, six birds on the second day, one bird on the third day, three birds on the fourth day and one bird on the fifth day.

Consistency of Responses and Novelty Syndromes

There was no evidence for significant repeatability in the number of approaches prior to first feed, to familiar seed [$R = 0$ (0 – 0.20), $P = 1$], with marginal evidence for novel seed [$R = 0.283$ (0 – 0.62), $P = 0.077$]. The cross study analysis for a potential novelty syndrome did not reveal a correlation between approach frequencies before first feed and any measures of spatial novelty reactions (**Table 1**).

Latency to first feed was moderately repeatable and significant for familiar seed [$R = 0.391$ (0.024 – 0.673), $P = 0.015$] but not for novel seed [$R = 0.17$ (0 – 0.57), $P = 0.189$]. Cross study Spearman correlations linking latency to feed with spatial novelty reactions were all non-significant, although we found one marginal and positive trend between the latency to first feed on familiar food and approach frequency to dense habitat (Spearman: $n = 31$, Corr Coef = 0.34, $P = 0.060$). Birds that were hesitant to enter unsuitable habitats tended to feed later on familiar food (**Table 1**).

Number of Approaches Before First Feed to Familiar and Novel Food

Results of the GLMMs for number of approaches are shown in **Table 2**. For model A, testing for effects of head color and seed type (**Table 2A**), there was a significant three-way interaction between food type, head color and experiment for number of approaches prior to first feed [GLMM: $n = 31$ birds (124 data points), LRT = 4.56, df = 1, $P = 0.033$; **Figure 2**] including a two-way interaction between food type and head color ($z = 2.43$, $P = 0.015$). Posthoc tests revealed that red-headed birds made significantly more approaches to novel food in experiment 1 than experiment 2 ($z = -4.901$, $P < 0.001$), whereas black-headed birds made significantly fewer approaches to novel food prior to first feed in experiment 1 than they did in experiment 2 ($z = 3.673$, $P < 0.001$). Also, red-headed birds tended to make more approaches to novel food before first feed than did black-headed birds in experiment 1 ($z = 9.911$, $P = 0.056$), while there was no difference in number of approaches to novel food before first feed between head morphs in experiment 2 ($z = -0.063$, $P = 0.95$).

Irrespective of head color, birds made more approach attempts toward novel than familiar food ($z = 2.47$, $P = 0.014$; **Figure 2**). There were no main effects of either head color, experiment or age.

Results for model B testing for social effects of own and partner head color on food neophobia are shown in **Table 2B**. There was no significant three-way interaction between food type, head color morph and partner head color. Removal of this term revealed a significant two-way interaction between head color morph and partner head color (GLMM: $n = 31$ (124 data points), LRT = 5.35, $P = 0.021$; **Figure 3**). *Post hoc* tests revealed that black-headed partners led to significantly more approach attempts in red-headed birds (mean 6.00 ± SE 2.11) than black-headed birds (mean 3.59 ± SE 0.91; $z = 10.478$, $P < 0.001$), whereas red-headed partners did not differentially impact number of approach attempts by red and black headed birds (red: mean 4.36 = SE 1.04; black: mean 4.85 = SE 1.39; $z = 1.591$, $P = 0.112$). The significant main effect of food type

TABLE 1 | Correlation between food neophobia measures and spatial novelty reactions.

	Approach frequency before entering open habitat		Latency to enter open habitat		Approach frequency before entering dense habitat		Latency to enter dense habitat	
	Corr Coef	***P*-value**	**Corr Coef**	***P*-value**	**Corr Coef**	***P*-value**	**Corr Coef**	***P*-value**
Approach frequency before first feed to familiar food	– 0.224	0.237	– 0.036	0.849	– 0.003	0.986	– 0.070	0.708
Latency to first feed on familiar food	– 0.183	0.325	– 0.087	0.641	*0.342*	*0.060*	0.070	0.707
Approach frequency before first feed to novel food	– 0.029	0.877	– 0.113	0.547	– 0.268	0.145	– 0.234	0.205
Latency to first feed on novel food	0.156	0.401	– 0.196	0.291	– 0.212	0.253	– 0.301	0.100

Italics: trend.

Two measures of food neophobia (approach frequency before first feed and latency to first feed on familiar and novel food) from experiment 1 were correlated with two measures of spatial novelty (approach frequency before first entry and latency to enter an open and dense habitat) from experiment 1 in Mettke-Hofmann et al. (2020). Results are from Spearman correlations (n = 31).

TABLE 2 | Results of the general linear mixed effects model on the number of approaches before first feed on familiar and novel food of Gouldian finches addressing **(A)** the effect of food type and color morphs (model A) and **(B)** social effects (model B). Only the final model of each analysis is shown. The reference modality is in parentheses.

A. Effects of food type and head color (model A)

		r^2m			r^2c	
Effective size		0.47			0.88	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	− 0.56	0.60	− 0.94	0.35	− 1.74	0.62
Key predictor						
Food type (novel)	1.25	0.51	2.47	0.01	0.26	2.25
Head color (red)	− 0.50	0.88	− 0.57	0.57	− 2.23	1.23
Controls						
Experiment	0.27	0.32	0.84	0.40	− 0.36	0.90
Age (older)	0.43	0.30	1.42	0.16	− 0.16	1.02
Interactions						
Food type × head color	1.80	0.74	2.43	0.02	0.35	3.26
Food type × experiment	0.15	0.30	0.48	0.63	− 0.45	0.74
experiment × head color	0.33	0.53	0.61	0.54	− 0.72	1.37
Food type × head color × experiment	− 0.99	0.46	− 2.17	0.03	− 1.89	− 0.10

B. Social effects (model B)

		r^2m			r^2c	
Effective size		0.48			0.88	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	0.04	0.54	0.08	0.93	− 1.02	1.11
Key Predictor						
Food type (novel)	1.65	0.11	14.80	<0.001	1.43	1.87
Head color (red)	0.79	0.40	1.98	0.05	0.01	1.57
Partner head color (red)	0.45	0.23	1.94	0.05	0.00	0.91
Controls						
Relative age (within pairs; younger)	− 0.43	0.25	− 1.71	0.09	− 0.93	0.06
Interactions						
Head color × partner head color	− 0.48	0.20	− 2.36	0.02	− 0.88	− 0.08

already reported in model 1 was again evident (LRT = 290.26, $P = 0.001$). There was no main effect of relative age within pair (LRT = 3.04, $P = 0.081$).

Latency to First Feed on Familiar and Novel Food

Results of the GLMMs for latency to first feed are shown in **Table 3**. For model A, testing for effects of head color and seed type (**Table 3A**), there were no significant interactions. There were significant main effects of food type (LRT = 57.09, df = 1, $P = 0.001$) and age (LRT = 5.25, df = 1, $P = 0.022$). Birds were faster to feed on familiar food (mean = 356 s ± 542 s) than on novel food (mean = 7202 s ± 10698 s). One-year old birds were faster to first feed, irrespective of food type (mean = 1272 ± 2949 s) than were older birds (mean = 4973 ± 9659 s).

Results for model B, testing for social effects, are shown in **Table 3B**. There were no significant interactions and no main effects of any of the variables associated with social context. The main effect of food type revealed in model A was retained (LRT = 55.78, df = 1, $P < 0.001$).

Rate of Incorporation of Novel Food Into the Diet

Consistency of Responses and Novelty Syndromes

Repeatability for feed frequency overall was moderate and significant ($R = 0.26$, $P < 0.001$), and present for both novel seed [$R = 0.384$ (0.18 – 0.47), $P < 0.001$] and familiar seed [$R = 0.361$ (0.196 – 0.509), $P < 0.001$]. In the cross species comparison some correlations were found. The frequencies of feeding visits to familiar food on day 1 and 5 were positively correlated with the approach frequency to open (suitable) habitat; birds with many approach attempts before entering the novel environment made more feeding visits to familiar food (**Table 4**). A similar trend was found for novel food on day 1. Moreover, feeding visits to novel food on day 5 were negatively correlated with the latency

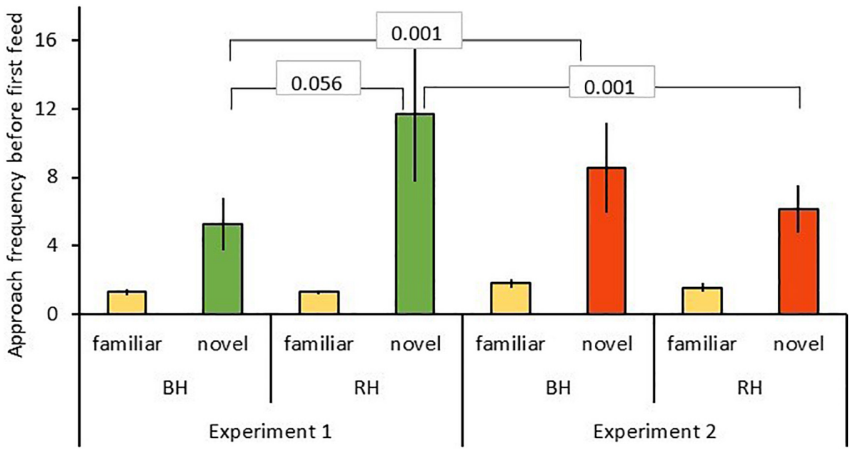

FIGURE 2 | Approach frequencies before first feed (mean ± SE) to familiar (beige) and novel food (green, red) in experiment 1 and 2 for red-headed (RH) and black-headed (BH) Gouldian finches. Numbers in the figure represent *p*-values.

to enter open (trend) and dense habitats. Birds that entered novel habitats sooner also made frequent visits to the novel food on day 5 (**Table 4**).

Rate of Incorporation of Novel Food

Results for rate of incorporation of food into the diet after the first feed are shown in **Table 5**. For model C, testing for effects of head color and seed type, there were significant three-way interactions between food type, head color and day (LRT = 6.85, df = 1, P = 0.009; **Table 5A** and **Figure 4**), and between food type, head color and experiment (LRT = 21.97, df = 1, $P < 0.001$; **Table 5A** and **Figure 5**). As part of the three-way interaction, there were significant two-way interactions between food type and head color (z = 3.82, $P < 0.001$), between food type and experiment (z = 5.60, $P < 0.001$) and between food type and day (z = 3.55, $P < 0.001$). Planned *post hoc* comparisons were conducted to explore the interaction between food type and day, for each head color separately. For red headed birds these revealed a significant increase in frequency of feeds on novel food between day 1 (mean = 6.46 ± 1.62 feeds) and day 5 (mean = 13.14 ± 2.65 feeds; V = 10, P = 0.025), with no difference in frequency of feeds on novel versus familiar food on day five (familiar mean = 14.93 ± 1.48 feeds; V = 62, P = 0.572). For black headed birds there was no increase in number of feeds on novel food from day 1 to 5 (V = 36.5, P = 0.190), and they continued to feed on familiar food significantly more often than novel food on day 5 (V = 127, P = 0.003; **Figure 4**).

The three-way interaction between food type, head color, and experiment was driven by the significantly lower number of feeding visits to familiar food by black-headed birds in experiment 1 as compared to red-headed birds (black: mean 12.3 ± SE 0.8; red: mean 16 ± SE 1.1; df = 153, t = –2.813, P = 0.006). This difference between head colors disappeared in experiment 2 (black: mean 17 ± SE 0.9; red: mean 17.7 ± SE 1.0; df = 153, t = –0.456, P = 0.649). In contrast, feeding visits to novel food by black-headed birds were significantly lower than by red-headed birds in both experiments (exp 1: df = 153, t = –3.200, P = 0.002; exp. 2: df = 168, t = –3.173, P = 0.002; **Figure 5**).

Furthermore, the main effect of age was significant (LRT = 6.57, df = 1, P = 0.010). One-year old birds generally visited the feeders (irrespective of type) more often (mean 16.34 ± SE 0.67) than older birds (mean 9.78 ± SE 0.47).

Results for model D, testing for social effects, are shown in **Table 5B**. There was a significant three-way interaction between seed type, head color and partner head color (LRT = 3.84, df = 1, P = 0.050). Red-headed birds made significantly more feeding visits to novel food than black-headed birds when partnered with a red-headed bird (red-headed bird: mean 11.76 ± 1.6; black-headed bird: mean 6.61 = 0.87; df = 1, t = –7.61, P = 0.006) but not when partnered with a black-headed bird (red-headed bird: mean 8.61 ± 1.04; black-headed bird: mean 6.36 ± 0.93; df = 153, t = –1.62, P = 0.107). Partner head color did not affect the number of visits of red-headed or black-headed birds to familiar food (red-headed partner: df = 153, t = –1.57, P = 0.119; black-headed partner: df = 153, t = –1.61, P = 0.110; **Figure 6**).

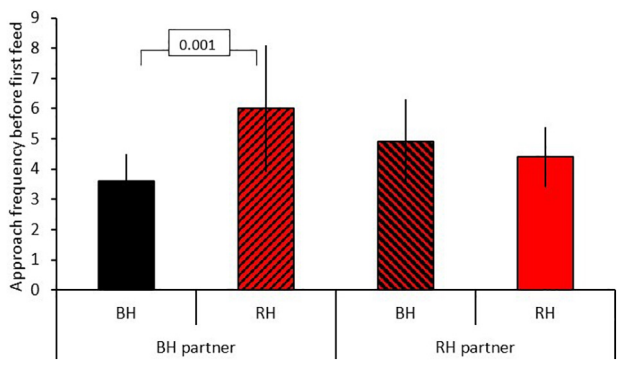

FIGURE 3 | Effect of partner head color on approach frequency before first feed (mean(SE) for red-headed (RH) and black-headed (BH) birds. Numbers in the figure represent p-values; black bars = black-headed pairs, hatched bars = mixed head color pairs, red bars = red-headed pairs.

TABLE 3 | Results of the linear mixed effects models on the latencies to first feed on familiar and novel food of Gouldian finches addressing **(A)** the relationship between food type and color morph (model A) and **(B)** social effects (model B). Only the final model for each analysis is shown. The reference modality is in parentheses.

A. Effects of food type and head color (model A)

	r^2m				r^2c	
Effective size	0.33				0.61	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	− 0.56	0.04	− 15.51	<0.001	− 0.63	− 0.49
Key predictor						
Food type (novel)	0.13	0.01	9.18	<0.001	0.10	0.16
Head color (red)	0.02	0.02	0.77	0.441	− 0.03	0.07
Controls						
Experiment	0.02	0.01	1.44	0.149	− 0.01	0.05
Age (older)	0.07	0.03	2.28	0.023	0.01	0.12

B. Social effects (model B)

	r^2m				r^2c	
Effective size	0.28				0.59	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	−0.50	0.05	− 10.16	<0.001	− 0.60	− 0.40
Key predictor						
Food type (novel)	0.13	0.01	9.03	<0.001	0.10	0.16
Head color (red)	0.01	0.03	0.19	0.851	− 0.05	0.06
Partner head color (red)	0.02	0.02	0.89	0.373	− 0.02	0.06
Controls						
Relative age (within pairs; younger)	0.00	0.03	0.16	0.874	− 0.05	0.05
Interactions						
Head color × partner head color	−0.00	0.03	− 0.16	0.874	− 0.05	0.05

TABLE 4 | Correlation between rate of incorporation of novel food into the diet and spatial novelty reactions.

	Approach frequency before entering open habitat		**Latency to enter open habitat**		**Approach frequency before entering dense habitat**		**Latency to enter dense habitat**	
	Corr Coef	***P*-value**	**Corr Coef**	***P*-value**	**Corr Coef**	***P*-value**	**Corr Coef**	***P*-value**
Frequency of feeding visits to familiar food day 1	**0.367**	**0.043**	–0.065	0.730	− 0.101	0.590	−0.106	0.571
Frequency of feeding visits to familiar food day 5	**0.399**	**0.026**	–0.009	0.961	0.050	0.788	−0.061	0.746
Frequency of feeding visits to novel food day 1	*0.348*	*0.055*	–0.062	0.742	− 0.116	0.534	0.051	0.787
Frequency of feeding visits to novel food day 5	0.012	0.947	*–0.341*	*0.061*	− 0.232	0.209	**–0.395**	**0.028**

Frequency of feeding visits to familiar and novel food after first feed in experiment 1 were correlated with two measures of spatial novelty (approach frequency before first entry and latency to enter an open and dense habitat) from experiment 1 in Mettke-Hofmann et al. (2020). Results are from Spearman correlations (n = 31).
Italics: trend; bold: significant.

DISCUSSION

We investigated whether novelty responses to food and acceptance of novel food into the diet are part of personality traits, whether these traits align with color morph and how group composition affects these responses in the food specialized Gouldian finch. We found that food neophobia and dietary conservatism were differentially expressed and were influenced by head color and group composition. Specifically, we found that birds' food neophobia was consistent in certain situations, and partially tied to their spatial novelty responses. Meanwhile, they exhibited clear individual consistency and a behavioral syndrome in dietary conservatism. Head color influenced birds' approach frequencies prior to their first feed and the rate of incorporation of novel food into the diet, further supporting that personalities are linked to head color morphs. Moreover, group

TABLE 5 | Results of the general linear mixed effects model on the frequency of feeding visits after first feed on familiar and novel food (rate of incorporation of novel food into the diet) of Gouldian finches addressing **(A)** the effect of food type and color morph (model C) and **(B)** social effects (model D). Only the final model of each analysis is shown. The reference modality is in parentheses.

A. Effects of food type and head color (model C)

	r^2m				r^2c	
Effective size	0.54				0.92	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	2.54	0.25	10.18	<0.001	2.05	3.03
Key Predictor						
Food type (novel)	− 1.60	0.15	− 10.56	<0.001	− 1.90	− 1.31
Head color (red)	− 0.07	0.33	− 0.20	0.841	− 0.71	0.58
Day	− 0.01	0.01	− 0.85	0.398	− 0.04	0.02
Controls						
Experiment	0.23	0.10	2.35	0.019	0.04	0.42
Age (older)	− 0.51	0.19	− 2.69	0.007	− 0.88	− 0.14
Latency to first feed	− 0.33	0.03	−10.74	<0.001	− 0.39	− 0.27
Interactions						
Food type × head color	0.78	0.20	3.82	<0.001	0.38	1.17
Food type × experiment	0.43	0.08	5.60	<0.001	0.28	0.58
Experiment × head color	0.09	0.18	0.47	0.641	− 0.27	0.44
Food type × day	0.09	0.03	3.55	<0.001	0.04	0.14
Head color × day	− 0.02	0.02	− 1.04	0.298	− 0.06	0.02
Food type × head color × experiment	− 0.48	0.10	− 4.68	<0.001	− 0.68	− 0.28
Food type × head color × day	0.09	0.04	2.62	0.009	0.02	0.16

B. Social effects (model D)

	r^2m				r^2c	
Effective size	0.24				0.95	
	Estimate	***SE***	***z*-value**	***P*-value**	**CI (2.5%)**	**CI (97.5%)**
(Intercept)	3.17	0.45	7.09	<0.001	2.30	4.05
Key predictor						
Food type (novel)	−0.64	0.05	− 12.00	<0.001	− 0.75	− 0.54
Head color (red)	−0.05	0.25	− 0.20	0.839	− 0.55	0.44
Partner head color (red)	0.28	0.20	1.42	0.155	− 0.11	0.67
Controls						
Relative age (within pairs; younger)	−0.13	0.22	− 0.59	0.558	− 0.55	0.30
Latency to first feed	−0.29	0.03	− 9.64	<0.001	− 0.35	− 0.23
Interactions						
Food type × head color	0.20	0.07	2.71	0.007	0.05	0.34
Food type × partner head color	−0.03	0.07	− 0.35	0.728	− 0.17	0.12
Head color × partner head color	−0.32	0.15	− 2.18	0.029	− 0.61	− 0.03
Food type × head color × partner head color	0.20	0.10	1.96	0.050	0.00	0.39

composition mattered. The presence of black-headed partners increased the approach frequency before first feed in red-headed but not in black-headed birds. Meanwhile the presence of red-headed partners increased the acceptance of novel food into the diet in other red-headed birds, but not in black-headed birds. On the species level, Gouldian finches were food neophobic and demonstrated dietary conservatism by making more approach attempts to novel food as compared to familiar food before first feed, sampling novel food later than familiar food and continuing to feed on familiar food more often than on novel food. Therefore, we found evidence for species-level dietary wariness.

Food Neophobia

We first predicted that individuals would be consistent in their food neophobic reaction. Individuals' neophobia reactions showed a weak trend to be consistent for approach frequencies to novel food before first feed and differed consistently in the latency

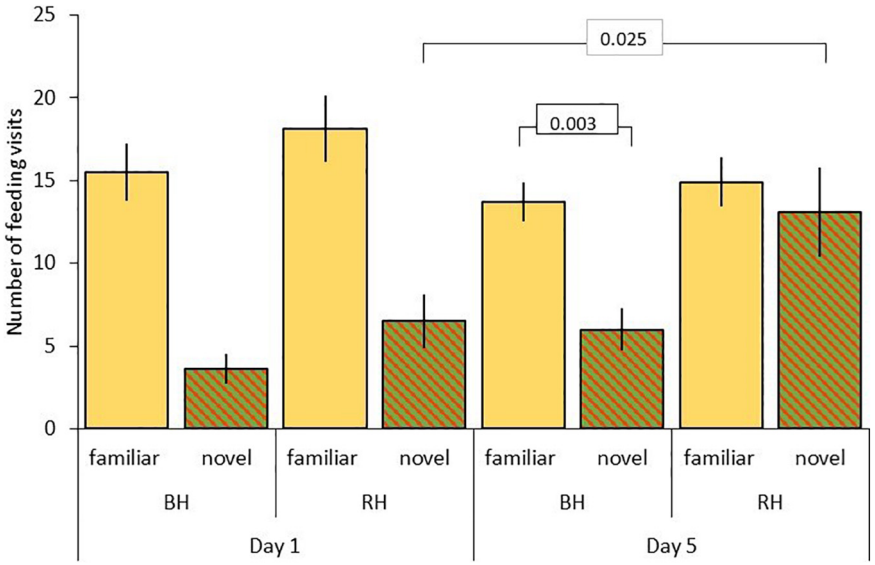

FIGURE 4 | Number of feeding visits after first feed (mean ± SE) to familiar (beige) and novel food (red/green hatched) on day 1 and 5 for red-headed (RH) and black-headed (BH) Gouldian finches. Numbers in the figure represent *p*-values.

to first feed on familiar food. This confirms our first prediction partly. Approach frequencies before first feed, particularly to novel food, are an indicator of fear as it reflects the conflict between the motivation to approach and sample the novel food and to avoid it (Mettke-Hofmann et al., 2009). Effects of the differently colored food in experiment 1 and 2 and social effects could have masked individual consistency. Birds were tested with partners of different head colors in the two experiments, which affected approach frequencies before first feed in both head colors differently. Testing birds in the same pairing may provide clearer results whether approach frequencies are indeed part of personality traits.

Latency to first feed on familiar food confirmed our prediction 1. This indicates that some individuals consistently approach and consume familiar food fast, whereas others consistently wait longer to consume familiar food when novel food is close by. This hints at a personality trait linked to food neophobia. The reason why we do not find similar consistent latencies to feed on novel food may be that Gouldian finches show conformity in risky and novel situations (King et al., 2015). Sampling novel food involves risk and individuals may align their response with others to reduce risk overwriting individual differences. Moreover, partner effects and the color of the novel food may have masked consistency. Other studies demonstrated consistency in food neophobia between breeding and non-breeding seasons in jackdaws (*Corvus monedula*; Greggor et al., 2016a). Similarly, Japanese macaques (*Macaca fuscata*) showed consistent food neophobia reactions (Arnaud et al., 2017).

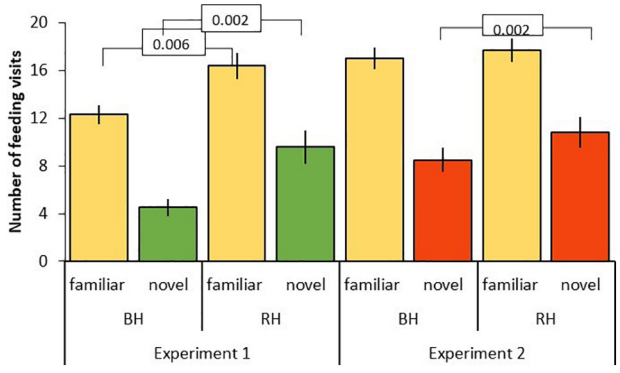

FIGURE 5 | Number of feeding visits after first feed (mean ± SE) to familiar (beige) and novel food (green, red) in experiment 1 and 2 for red-headed (RH) and black-headed (BH) Gouldian finches. Numbers in the figure represent *p*-values.

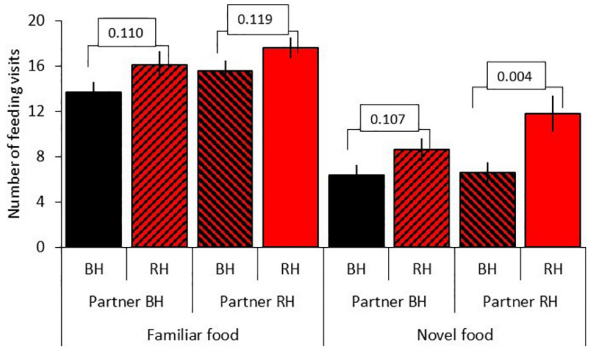

FIGURE 6 | Effect of partner head color on the number of feeding visits after first feed (mean = SE) to familiar and novel food for red-headed (RH) and black-headed (BH) Gouldian finches. Numbers in the figure represent *p*-values; black bars = black-headed pairs, hatched bars = mixed head color pairs, red bars = red-headed pairs.

Approach frequencies toward novel food before first feed differed between head color morphs. Red-headed birds made more approach attempts toward novel food in experiment 1 as compared to experiment 2, whereas this was reversed in black-headed birds. Moreover, red-headed birds tended to have more approach attempts toward novel food than black-headed birds in experiment 1 but not within experiment 2. This indicates that red-headed birds were more drawn to the novel food (food neophilia) but also reluctant to try it out (food neophobia) resulting in more approach attempts in experiment 1 reflecting the conflict between approach and avoidance (Mettke-Hofmann et al., 2009). Black-headed birds, in contrast, had fewer approaches, possibly reflecting less interest in the novel food (neophilia) but also less fear of the novel food (neophobia). This mirrors similar results in a novel spatial context with red-headed birds showing more interest in novel environments than black-headed birds (Mettke-Hofmann et al., 2020). Overall, this contradicts prediction 2, which expected black-headed birds to be more neophobic. As approach attempts to novel food tended to be repeatable, this may be another trait describing differences in personality between head colors. However, more research is needed to fully confirm this.

Despite latency to first feed on familiar food varying consistently among individuals, responses were not linked to head color. Likewise, latencies to first feed on novel food were unrelated to morph. This again contrasts with prediction 2 since individuals do not signal their food neophobia to others or others cannot use head color as a proxy for food neophobia in their peers. This is in line with findings regarding object neophobia in this species, which also showed no relationship to head color (Mettke-Hofmann, 2012; Williams et al., 2012). Signaling personality traits may not always be beneficial (Wolf et al., 2011). While, e.g., signaling aggression in conflict situations reduces agonistic encounters (Pryke, 2009), signaling food neophobia or the lack of it could attract competitors to newly encountered resources in socially foraging birds (Wolf et al., 2011).

There was no indication of a novelty syndrome between approach frequency before first feed and any measures of spatial neophobia. While we did find a positive trend between latency to first feed and latencies to enter unsuitable habitats, these effects were marginal and only slightly support prediction 3. More research is needed into how different novelty reactions are linked with each other.

In mixed head color pairs, black-headed partners induced a higher approach frequency before first feed in red-headed birds, whereas red-headed partners had no effect on black-headed birds. While this confirms that group composition has an effect, as we had predicted (prediction 4), the influence of partner head color did not manifest itself according to any of our predicted scenarios. That red-headed birds become more hesitant to approach food in the presence of black-headed birds is surprising given their higher aggression and ability to displace black-headed birds within competitive scenarios where food is a limited resource (Pryke and Griffith, 2006; Pryke, 2007; Williams et al., 2012). While this result combines approach attempts to both, familiar and novel food, all birds made more approach attempts to novel food (see **Figure 2**) and differences seem to be primarily down to approach attempts to novel food (see **Supplementary Material S3**). The following explanation is therefore suggested. The disinterest in novel food by the black-headed partner may have increased fear and cautiousness in red-headed birds. In combination with their interest in the novel food, this increased their approach attempts. Interestingly, black-headed partners had the same effect on red-headed birds in a novel spatial context (Mettke-Hofmann et al., 2020).

On the species level, Gouldian finches showed clear food neophobia by making on average more approach attempts before first feed to novel than familiar food indicating fear of the novel food (Malmkvist et al., 2003; Mettke-Hofmann et al., 2009). They also fed on the familiar food sooner than the novel food. Both findings confirm our fifth prediction (species-level dietary wariness). The results are in line with other studies demonstrating food neophobia in a variety of species (humans, Cooke et al., 2007; Knaapila et al., 2007, non-human primates, Johnson, 2000; Visalberghi and Addessi, 2000; Visalberghi et al., 2003; Gustafsson et al., 2014; Forss et al., 2019, rodents, Hall et al., 1997; Lin et al., 2009; Modlinska et al., 2015, carnivores, Malmkvist et al., 2003 and birds Marples et al., 1998; Kelly and Marples, 2004; Camín et al., 2016). Alternatively, due to the 1-h food deprivation prior to the experiment, birds may have preferred the familiar option over the unfamiliar one to refuel energy reserves before sampling the novel food (Inglis and Ferguson, 1986; Beaulieu and Schaefer, 2014). However, the extended period of avoiding novel food in several individuals in our study indicates a stronger effect of food neophobia than just the desire to refuel energy reserves quickly. In any way, the birds must have perceived the novel food as riskier, otherwise they could have fed from both food types equally fast.

Gouldian finches are specialist granivores, heavily relying upon annual Sorghum species during the breeding season (Brazill-Boast et al., 2011) and on perennial grasses during the wet season (Weier et al., 2016). They have been shown to suffer reduction in body condition and increased stress levels possibly linked to food shortage during the wet season (Maute et al., 2013; Legge et al., 2015), due to intense fire regimes preventing the growth and seeding of perennial grasses (Weier et al., 2016, 2018). Such physiological changes have not been observed in closely related sympatric species that are more diet generalists (Maute et al., 2013). This suggests that Gouldian finches on the species level are reluctant or unable to switch to other seed types. Our study indicates that one factor responsible for this lack of plasticity is food neophobia, since some birds hesitated up to 5 days to sample the novel food. Similar patterns have been found in other food specialized species (Beissinger et al., 1994). High food neophobia may prevent specialized birds from ingesting unsuitable food, which is in line with the Neophobia Threshold hypothesis (Greenberg, 1983). However, future studies should investigate food neophobia in closely related, generalist foragers to support this further. Interestingly, Addessi et al. (2007) found that common marmosets (*Callithrix jacchus*) who are dietary generalists and occupy small home ranges were less hesitant to sample novel food than Goeldi's monkeys (*Callimico goeldii*), who are dietary specialists and utilize a large home range. The marmosets seem to be willing to exploit new resources

in their restricted home range, whereas the monkeys were roaming around widely to find familiar food. Gouldian finches are nomadic outside the breeding season tracking suitable food resources (Woinarski and Tidemann, 1992; Dostine et al., 2001) and may follow a similar roaming strategy as the Goeldi's monkeys.

Incorporation of Novel Food Into the Diet

Individuals showed consistent among individual differences in incorporating novel food into the diet with some individuals being adventurous consumers and others dietary conservatives. Only few other studies have so far shown individual consistency in accepting novel food (Thomas et al., 2010; but see Marples et al., 1998; Prasher et al., 2019). This finding is in support of prediction 1 on top of the consistent differences in food neophobia latencies toward familiar food discussed earlier. Additionally, food acceptance correlated with novelty responses to enter an unfamiliar space (Mettke-Hofmann et al., 2020) in support of a novelty syndrome confirming prediction 3. Individuals that had entered a novel environment quickly, were those that ate less of the familiar food and accepted the novel food by day 5. The combination of a high willingness to enter novel environments and accepting novel food is advantageous as mobile individuals are likely to encounter novel food resources. The novelty syndrome links to the existence of dispersal syndromes. Dispersing individuals in a population are often more spatially explorative, less social and more aggressive than philopatric individuals (Cote et al., 2010; Paulauskaite et al., 2010; Ciani and Capillupi, 2011). They may also be adventurous consumers as it helps individuals to quickly adapt to new resources. The findings are also in support of a resident and nomadic cognitive strategy (Mettke-Hofmann et al., 2020), particularly as red-headed birds turned out to be adventurous consumers and black-headed birds dietary conservatives, which continued to avoid the novel food. In earlier experiments with the same individuals, red-headed birds were more willing to enter unfamiliar environments (Mettke-Hofmann et al., 2020). Therefore, the willingness to accept novel food may be part of the personality syndrome characterizing the two different head colors (Williams et al., 2012) and is in support of prediction 2. Unlike for food neophobia, group members can use head color as a proxy for the willingness to accept novel food. This may help spread acceptance of novel food in a group. Wolf et al. (2011) suggested that signaling traits is beneficial when coordination and collaboration is important. More hesitant individuals could particularly pay attention to more adventurous individuals.

Head color indeed affected the rate of incorporation of novel food into the diet in others, although in the opposite way then laid out above. While black-headed birds' willingness to incorporate the novel food was unaffected by their partner, red-headed birds became slower in accepting novel food into their diet when paired with a black-headed partner as compared when paired with a red-headed partner. The persistent avoidance of the novel food in black-headed birds likely made the red-headed birds more cautious. This is largely in line with prediction 4 expecting pure red-headed pairings being fastest in accepting novel food and black-headed birds slowing down red-headed ones in mixed pairs. However, in contrast to our prediction, pure black-headed pairings were not slower than mixed pairs. The reason for this result seems to be that black-headed birds do not change their behavior in relation to others. Similar results have been found for object exploration and risk-taking when black-headed birds did not conform when paired with red-headed birds (King et al., 2015). Red-headed birds, in contrast, seem to pay attention to others and become more careful. This is in line with similar effects when entering novel environments; black-headed partners as compared to red-headed partners led to more approach attempts before entering a novel environment in red-headed birds (Mettke-Hofmann et al., 2020). The presence of black-headed birds seemed to act as a cautionary note to red-headed birds, rather them being a motivator for black-headed birds to accept novel food. More research is needed to understand this relationship and the consequences on the species level further.

On the species level, Gouldian finches were not only food neophobic but also hesitated to incorporate novel food into their diet, demonstrating dietary conservatism confirming the second part of prediction 5. This foraging response can be attributed to their food specialism (Brazill-Boast et al., 2011) and does not seem to be the response to interspecific competition (Jessopp et al., 2020). Similar dietary conservatism has been found in the specialized snail kite (Beissinger et al., 1994).

Dietary Wariness in a Changing World

As a dietary specialist with strong dietary wariness Gouldian finches may struggle in a changing world. This is evidenced already in earlier work as Gouldian finches experience reduced body condition and increased stress levels during times of food shortage in the non-breeding season (Maute et al., 2013; Legge et al., 2015). However, we currently do not know whether head colors are differently affected. From our results we would predict that black-headed birds suffer more than red-headed birds. An experimental study manipulating protein content before and during breeding in captive Gouldian finches showed that red-headed birds need a higher protein-diet than black-headed birds to maintain body condition and raise their young successfully (Pryke et al., 2012). Being more open to accept novel food would help red-headed birds to gain access to a high-protein diet during breeding. This may carry over into the non-breeding season and allow red-headed birds to utilize unfamiliar food during times of food shortage.

While the species as a whole is dietary wary, not all Gouldian finches are dietary conservative and consistent among individual variation in their willingness to accept novel food may help to adapt to a changing world. Indeed, Forsman et al. (2008) suggested that polymorphism in traits is advantageous when encountering environmental change as individuals use different resources and strategies. Groups of mixed personalities have been shown to approach novel feeding situations faster and have improved patch exploitation and group cohesion (Dyer et al., 2009; Aplin et al., 2014). While mixed groups in the current study were not the fastest in incorporating novel food, they may maximize use of existing and newly occurring food with black-headed birds preferring the familiar food and red-headed birds diversifying by incorporating novel food. The red-headed birds'

responsiveness to other group members may slow them down enough that black-headed birds can eventually catch up and start exploiting this new food source. Future studies should determine when, if at all, black-headed birds incorporate novel food into their diet and whether mixed groups speed up this process.

In the wild, black-headed birds make up 70% in most populations (Kim et al., 2019). This means that dietary conservatives are by far outnumbering adventurous consumers. However, a few individuals can affect group decisions, e.g., whether and where to move (Couzin et al., 2005). Potentially, a small number of adventurous consumers is enough to slowly spread the use of new food types. More research is needed to better understand how group composition affects individual preferences.

Overall, the results indicate that food neophobia and in particular dietary conservatism contribute to maintaining a specialist's diet. Moreover, they show that even in a food specialized species consistent among individual variation exists in response to novel food with some individuals being adventurous consumers. This individual variation may help adapting to new resources. In our specific case, individual differences in accepting novel food into the diet were linked to color morph, adding to the increasing evidence that color morphs respond differently to environmental challenges (Mateos-Gonzalez and Senar, 2012; Schweitzer et al., 2015; Mettke-Hofmann et al., 2020). Both, individual and morph-specific differences in response to environmental challenges have rarely been considered in conservation-oriented studies (but see Kelleher et al., 2018). However, they may be an important component of a species' survival chances.

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 reviewed and approved by LJMU Ethics Committee.

AUTHOR CONTRIBUTIONS

GE conducted all experiments, transcribed all data, did initial analyses, and co-wrote the sections "Abstract, Introduction, Materials and Methods, and Discussion." EB analyzed the data for the manuscript, co-wrote the "Materials and Methods" section and wrote the "Results" section. AG contributed to the experimental design and initial analyses and gave important feedback on the manuscript. CM-H came up with the design, advised on data collection and analyses, and co-wrote the sections "Abstract, Introduction, and Discussion." All the authors contributed to the manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.772812/full#supplementary-material

Supplementary Material S1 | Food Neophobia raw dataset.

Supplementary Material S2 | Dietary Conservatism raw dataset.

Supplementary Material S3 | Approach frequencies (mean ± SE) before first feed to familiar and novel food considering effects of partner head colors on black-headed (BH) and red-headed (RH) birds. Black bars: pure black-headed pairs; hatched red/black bars: mixed head color pairs; red bars: pure red-headed pairs.

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Variation in Hematological Indices, Oxidative Stress, and Immune Function Among Male Song Sparrows From Rural and Low-Density Urban Habitats

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A central theme in the field of ecology is understanding how environmental variables influence a species' distribution. In the last 20 years, there has been particular attention given to understanding adaptive physiological traits that allow some species to persist in urban environments. However, there is no clear consensus on how urbanization influences physiology, and it is unclear whether physiological differences in urban birds are directly linked to adverse outcomes or are representative of urban birds adaptively responding to novel environmental variables. Moreover, though low-density suburban development is the fastest advancing form of urbanization, most studies have focused on animals inhabiting high intensity urban habitats. In this study, we measured a suite of physiological variables that reflect condition and immune function in male song sparrows (*Melospiza melodia*) from rural and suburban habitats. Specifically, we measured hematological indices [packed cell volume (PCV), hemoglobin concentration, mean corpuscular hemoglobin concentration (MCHC)], circulating glutathione (total, reduced, and oxidized), oxidative damage (d-ROM concentration), antioxidant capacity, and components of the innate immune system [bacteria killing ability (BKA), white blood cell counts]. We also measured whole-animal indices of health, including body condition (scaled mass index length) and furcular fat. Song sparrows inhabiting suburban environments exhibited lower hemoglobin and MCHC, but higher body condition and furcular fat scores. Additionally, suburban birds had higher heterophil counts and lower lymphocyte counts, but there were no differences in heterophil:lymphocyte ratio or BKA between suburban and rural birds. PCV, glutathione concentrations, and oxidative damage did not differ between suburban and rural sparrows. Overall, suburban birds did not exhibit physiological responses suggestive of adverse outcomes. Rather, there is some evidence that sparrows from rural and suburban habitats exhibit phenotypic

differences in energy storage and metabolic demand, which may be related to behavioral differences previously observed in sparrows from these populations. Furthermore, this study highlights the need for measuring multiple markers of physiology across different types of urban development to accurately assess the effects of urbanization on wildlife.

Keywords: urbanization, avian physiology, oxidative stress, immune function, hemoglobin

INTRODUCTION

Urbanization dramatically restructures ecosystems by introducing novel environmental variables (Marzluff, 2001; Hobbs et al., 2006) and poses one of the largest threats to wildlife (Aronson et al., 2014). Remarkably, in some species, individuals are able to persist in both urban and rural habitats (i.e., "urban adapters"; Blair, 1996; McKinney, 2006), yet the underlying physiological traits that allow individuals of the same species to live in disparate habitats remain unclear. Animals inhabiting urban environments must cope with anthropogenic light and noise, increased exposure to toxicants, altered predator and prey communities, and shifts in disease exposure (Marzluff, 2001; Isaksson, 2018). While some animals are able to thrive under these conditions (i.e., "urban exploiters"; Blair, 1996; McKinney, 2006), there is an underlying assumption that inhabiting urban habitats is costly for most individuals (Birnie-Gauvin et al., 2016; Murray et al., 2019). Several studies have examined the physiological and fitness consequences of urbanization by comparing urban and rural dwelling birds within species and found urban birds exhibit lower body condition (Capilla-Lasheras et al., 2017; Murray et al., 2019) and reduced reproductive success (Chatelain et al., 2021). However, there are also cases where birds inhabiting urban environments exhibit higher body condition (Auman et al., 2008; Minias, 2016), reproductive success (Lane et al., unpublished data) and survival (Møller, 2009b; Phillips et al., 2018) compared to rural birds of the same species. These contradictory results among previous studies highlight the need to understand the physiological processes underlying broader condition and fitness outcomes for individuals living in urban environments (Isaksson, 2015; Ouyang et al., 2018).

A range of physiological mechanisms allow birds to cope with varying environmental conditions, but such mechanisms may carry fitness costs or result in trade-offs with other physiological processes. Consequently, the strongest studies measure several "biomarkers" of physiological condition to more accurately assess costs or benefits of dwelling in urban habitats (e.g., Bókony et al., 2012; Ibáñez-Álamo et al., 2020). Additionally, while it is often assumed that physiological differences among rural and urban birds are directly or indirectly linked to adverse health and fitness outcomes, it is also possible that physiological differences are indicative of urban birds adaptively responding to novel environmental variables (Isaksson, 2015). Therefore, to accurately characterize physiological responses to urbanization and determine if they reflect costs or adaptive responses requires examining multiple physiological variables that reflect a range of processes, including cellular damage, metabolic performance, and immune function.

Previous studies indicate birds inhabiting urban environments often exhibit higher oxidative stress, shifts in hematological variables, and altered immune function (Isaksson, 2015, 2018). Oxidative stress generally describes an imbalance between pro-oxidants (e.g., reactive oxygen and nitrogen species) and antioxidants [e.g., glutathione (GSH)] (Costantini, 2008), and is known to disrupt a myriad of physiological processes, including damage to red blood cell membranes and denaturation of hemoglobin molecules (Mohanty et al., 2014). In some cases, birds inhabiting urban environments exhibit lower packed cell volume (PCV; i.e., hematocrit) and hemoglobin concentrations (Llacuna et al., 1996; Cid et al., 2018), and shifts in these hematological indices may indicate adverse health outcomes in urban dwelling birds associated with increased oxidative stress (Minias, 2015; Johnstone et al., 2017). Alternatively, lower PCV and hemoglobin concentration in urban birds may reflect different metabolic demands related to habitat-specific differences in behaviors (Lowry et al., 2013; Charmantier et al., 2017). That is, in some cases, urban dwelling birds have smaller territory size, are less neophobic, and have shorter flight initiation distances (Senar et al., 2017; Juárez et al., 2020; Fossett and Hyman, 2021). These shifts in behavior may reduce aerobic requirements and thus hematological indices. The fact that urban and rural birds often differ in body condition, which is a rough of estimate of a bird's total energy reserve, further suggests urban and rural dwelling birds experience different energetic demands (Capilla-Lasheras et al., 2017; Phillips et al., 2018).

Shifts in energy budgets and higher oxidative stress in urban birds also may be associated with altered immune function (Watson et al., 2017; Cummings et al., 2020a,b). Indeed, urban dwelling birds tend to exhibit infection more often than rural birds, which could cause or be the result of altered immune function (Hamer et al., 2012; Bichet et al., 2013; Giraudeau et al., 2014; Rouffaer et al., 2017; Jiménez-Peñuela et al., 2019; Sykes et al., 2021). Interspecific comparisons among urban and rural dwelling avifauna indicate urban birds cope with increased disease exposure by investing in a more robust immune system (Møller, 2009b). Although investment in immune function may improve disease defense, chronic immune activation in urban habitats has been shown to occur at a cost to body condition (Capilla-Lasheras et al., 2017; but see Merrill et al., 2019). Taken as a whole, there is strong evidence that physiological responses to urbanization can lead to trade-offs that generate distinct urban and rural phenotypes (Isaksson, 2018). Nonetheless, determining if physiological responses to urbanization reflect fitness costs or adaptive coping requires further investigation (Isaksson, 2015).

In particular, it is critical to understand the effects of low-density urban environments on avian physiology because the main driver of urbanization globally is the expansion of small

and medium sized cities (McKinney, 2002; Fragkias et al., 2013). While most existing studies on the effects urbanization on avian physiology focus on comparisons among birds from rural and highly urbanized environments, prior work has shown that the impact of urbanization on avian richness and abundance can vary within and among urban areas depending on the intensity and recency of urbanization (MacGregor-Fors and Schondube, 2011; Ferenc et al., 2014; Evans et al., 2015, 2018). Given the rate of low density suburban expansion is predicted to increase, it is critical to examine physiological differences between rural birds and birds inhabiting moderately urbanized environments in order to predict how suburban expansion will impact avian populations (Marzluff, 2001; Isaksson, 2015).

In this study, we examined antioxidant capacity and oxidative damage, hematological indices, immune endpoints, and body condition in male song sparrows (*Melospiza melodia*) living across replicate suburban and rural study sites (see section "Site Selection"; **Supplementary Table 1**). Song sparrows are a common songbird native to North America and are an excellent model to study physiological differences across urban-rural gradients because they are considered urban adapters and are present in both rural and urban habitats. While our previous studies have detected consistent behavioral differences between rural and suburban song sparrows (Davies and Sewall, 2016; Davies et al., 2018), hormone concentrations to not reliably differ across habitats (Beck et al., 2018; Lane et al., 2021), suggesting other physiological variables may be associated with behavioral differences between rural and suburban dwelling birds. Additionally, there is no evidence of genetic differences between rural and suburban populations in our song sparrow system (Brewer et al., 2020). We predicted suburban birds would exhibit higher oxidative stress, lower PCV and hemoglobin, greater immune activation, and lower body condition.

MATERIALS AND METHODS

Site Selection

We sampled male song sparrows from three rural and three suburban field sites in and around the low-density cities of Blacksburg (human population: 44,074; human density: 1,390 per square km) and Radford (human population: 18,255; human density: 1,169 per square km) in Montgomery County, VA, United States (U.S. Census Bureau, 2021). We previously characterized the urbanization level of field sites using a technique described by Seress et al. (2014), whereby we divided an aerial image of the 1 km^2 area around each study site into 100 m × 100 m cells and scored the abundance of vegetation, buildings, and paved surfaces, such as roads and parking lots, in each cell. From these cell scores, we calculated the following summary land-cover measures for each study site: mean building density score, number of cells with high building density (>50% cover), number of cells with paved surfaces, mean vegetation density score, and number of cells with high vegetation density (>50% cover). We then used the PC1 score from a principal components analysis (PCA) of these landscape variable to calculate an "urbanization index" [see Seress et al. (2014) and Davies et al. (2018) for method validation and further details on site selection and characteristics]. In this study, we described sites with an urbanization index >3.0 as "suburban" and sites with an urbanization index <−1.70 as "rural" (**Supplementary Table 1**), though it is worth emphasizing that urbanization index is a continuous variable.

Sample Collection

We sampled a total of 136 male song sparrows from suburban and rural sites over 3 years (**Supplementary Table 1**). In a separate study, we were examining nest attendance behavior and reproductive physiology of female song sparrows in our study population; therefore, we only sampled male sparrows in an effort to avoid interfering with concurrent studies by repeatedly sampling female song sparrows. We captured male song sparrows during the breeding season (April–June) by playing conspecific calls at the center of previously mapped territories to lure males into mist nets [see Hyman et al. (2004) for how male territories were defined). All captures occurred between 500 and 1,200 h. We collected 150–175 μl of blood from each male sparrow (mass: 21.16 ± 1.09 mean ± SD) *via* venipunction of the basilar vein using a 26-gauge needles and heparinized capillary tubes. After blood sampling, we collected morphological measurements to determine body condition (i.e., scaled mass index; see section "Scaled Mass Index") We measured hemoglobin concentration and prepared blood smears in the field immediately after blood collection (see section "Hematological Indices"). We stored remaining blood on ice until we returned to the laboratory to further prepare blood for later analysis. An aliquot of whole blood was frozen at −80°C for later analysis of glutathione (see section "Oxidative Stress"). We centrifuged hematocrit tubes to separate plasma and measured PCV (see section "Hematological Indices"), then stored plasma at −80°C until analysis of oxidative damage and antioxidant capacity (see section "Oxidative Stress") and bacteria killing ability (BKA) (see section "Bacteria Killing Ability"). Data were collected opportunistically while conducting a separate concurrent study of female song sparrows, thus sample sizes varied across years. We collected plasma for BKA from 20 rural and 41 suburban males in year 2016. We collected plasma for d-ROMs and antioxidant capacity measurements from 15 rural and 20 suburban males in year 2017. We collected hematological indices, blood smears, glutathione concentrations, and scaled mass index from 20 rural and 20 suburban males in year 2020. We also measured furcular fat (scored 0–3) for birds collected during each field season. Researchers adhered to social distancing and safety precautions while collecting data for the 2020 field season during the COVID-19 global health crisis. Study design and methods were approved by the Virginia Tech Institutional Animal Care and Use Committee; all animals were immediately released after blood collection and were in good health.

Scaled Mass Index

To assess body condition, we calculated scaled mass index according to Peig and Green (2009). The scaled mass index has been proposed as a more robust estimation of body condition because it accounts for a changing relationship between mass

and body length as body size changes during growth (Peig and Green, 2010). Following Peig and Green (2009), we conducted Pearson correlations between mass and length measurements (head length, bill width, bill length, tarsus length, wing chord, and tail length) to determine which length measurement was the best predictor of body mass. For song sparrows in this study, wing chord was the best predictor of body mass ($R = 0.50$), therefore we used this variable in the calculation of scaled mass index.

Hematological Indices

We measured total hemoglobin in whole blood (∼5 μl) in duplicate using the HemoCue system (Ängelholm, Sweden) and used the mean of replicate measurements (intraassay variation: 2.19%) in the statistical analysis. We measured PCV as the percent red blood cells v/v whole blood. Additionally, we calculated mean corpuscular hemoglobin concentration (MCHC; i.e., amount of hemoglobin per red blood cell volume) as the quotient of hemoglobin concentration divided by PCV.

To count white blood cells, we stained blood smears with the JorVet Dip Quick Stain Kit (Jorgensen Labs, Loveland, CO, United States), and a single observer determined white blood cell differentials for each bird by examining at least 100 leukocytes at 100× magnification and counting the number of lymphocytes, heterophils, monocytes, and eosinophils (sensu Ots et al., 1998; Ewenson et al., 2001; Davis et al., 2004). We calculated heterophil:lymphocyte (H:L) ratio for each bird from the white blood cell differential.

Oxidative Stress

The concentrations of total GSH (tGSH) and free GSH in whole blood were measured using the DetectX Glutathione fluorescent detection kit (Arbor Assays, Inc., Ann Arbor, MI, United States) and following the manufacturer's instructions. Briefly, whole blood was diluted 1:2 with equal parts 5% sulfosalicylic acid, then kept on ice until assay buffer and sample diluent were added to increase the dilution to 1:300, which we validated as the optimal dilution for our study species. Diluted samples were transferred to a flat-bottom 96-well plate. Each plate contained duplicate 50 μl standards, controls, and 1:300 diluted samples. ThioStar (25 μl) reagent was added to each well, after which the plate was lightly tapped and incubated for 15 min. Free GSH concentration was measured fluorometrically at excitation/emission of 405/510 nm using a Tecan Infinite M200 microplate reader. After measuring free GSH, we measured tGSH by adding 25 μl of reaction mixture provided by the manufacturer to all wells. We then tapped the plate gently to mix constituents, incubated for 15 min, and measured fluorescence a second time at excitation/emission of 405/510 nm. We calculated the mean for duplicate sample results for both tGSH and free GSH. The intra- and interassay variations were 2.52–4.5 and 4.39%, respectively. We calculated oxidized GSH (GSSG) by subtracting free GSH from tGSH, then divided the result by two, per the manufacturer's instructions. Additionally, we calculated the ratio of GSH:GSSG.

We quantified two aspects of oxidative status using d-ROMs and OXY-Adsorbent kits (Diacron; Grosseto, Italy). The d-ROMs kit measures concentrations of reactive oxygen metabolites in plasma and is a measure of the amount of oxidative damage an individual has sustained. For this assay, we added 10 μl of plasma to each well followed by 200 μl of reagent mix. The plate was incubated for 1 min in the plate reader before being read. Second, we quantified the ability of the plasma antioxidant barrier to neutralize the oxidative action of hypochlorous acid (HOCl) using the Oxy-Adsorbent kit. We diluted plasma 1:100 with distilled water and then added 5 μl of diluted plasma to each sample well followed by 200 μl of HOCl solution. The plate was gently rocked while incubating at 37°C for 10 min after which we added 5 μl of chromagen solution to each well and mixed the wells with additional pipetting. We included blanks and calibration standards in triplicate on the plates for both types of assays. For both assays, we used a microplate reader (BioTek Synergy HTX) set to 37°C to measure sample absorbance at 505 nm and the plates were read kinetically once per minute for a total of 10 min. For both assays, we ran 20 samples in duplicate per plate and used the average of these in the analysis. The intraassay variation for the d-ROMs and Oxy adsorbent assays were 2.42 and 12.86%, respectively.

Bacteria Killing Ability

We first optimized the assay using a pooled aliquot of six plasma samples collected in the field from adult males in the previous 48 h. Plasma was diluted 1:5, 1:10, and 1:20 in sterile PBS and mixed with *Escherichia coli* (ATCC 8739, E^{power} microorganisms; Microbiologics® St. Cloud, MN, United States) at concentrations of 10^4, 10^5, and 10^6 colony forming units. Samples were incubated for 30 min at 37°C after which we added 250 μl of Tryptic soy broth (TSB, Sigma-Aldrich, St. Louis, MO, United States) to each sample. Samples were incubated at 37°C and the absorbance recorded after 8, 12, and 24 h of incubation. Samples were vigorously vortexed and absorbance quantified using a NanoDrop Spectrophotometer (ND-2000, Thermo Scientific, Pittsburgh, PA, United States) at OD300. The absorbance of each sample and the positive controls were each averaged and used to calculate the proportion of bacteria killed as 1-(sample absorbance/positive control absorbance). We found that the 1:20 plasma dilution with 10^5 *E. coli* concentration and 8 h of incubation resulted in approximately 50% bacteria killing. Following optimization, we evaluated bactericidal capacity of samples from individual males in triplicate (intraassay variation: 12.90%), and calculated the killing capacity using the average absorbance from triplicate measurements.

Statistics

We used R (Version 4.0.2; R Core Team, 2021) to statistically analyze whether physiological variables differed between rural and suburban birds. Many of the physiological traits we measured can fluctuate throughout the day and over the breeding season, thus Julian date and time of day birds were sampled are included as covariates in all models. We analyzed the effect of habitat on scaled mass index using a linear model (LM), we then included scaled mass index as a covariate in all models of physiological variables. To assess differences in fat score across habitats, we used a generalized linear model (GLM), with a Gamma distribution and included sample year as a

covariate. We used LMs to separately analyze the effect of habitat on PCV, hemoglobin, tGSH, free GSH, GSSG, and GSSG:tGSH. To analyze heterophil, lymphocyte, and monocyte counts, we used GLMs with a Poisson distribution for count data. For BKA, we arcsin square-root transformed *E. coli* counts to meet the assumption of normality, then compared habitat differences using a GLM with a Gamma distribution. We analyzed HOCl neutralization and d-ROMs data using separate GLMs, with a Gamma distribution. For both HOCl neutralization and d-ROMs data, we log-transformed data to meet assumptions of normality. All models were additive. Finally, we tested for correlations among physiological variables for data collected from the same individuals during the 2020 field season. BKA, d-ROMs, and HOCl neutralization were not included in correlation analyses because these data were collected during separate field seasons.

RESULTS

Scaled Mass Index and Fat Score

Scaled mass index was higher in birds collected from suburban sites compared to birds from rural sites during the 2020 season (**Figure 1A**; $|t| = 2.05$, $p = 0.048$) but did not vary within the season or by time of day (**Table 1**). Additionally, across all field seasons, suburban birds had a higher fat score compared to rural birds (**Figure 1B**; $|t| = 2.03$, $p = 0.04$). Regardless of habitat, fat score increased as the breeding season progressed ($|t| = 2.00$,

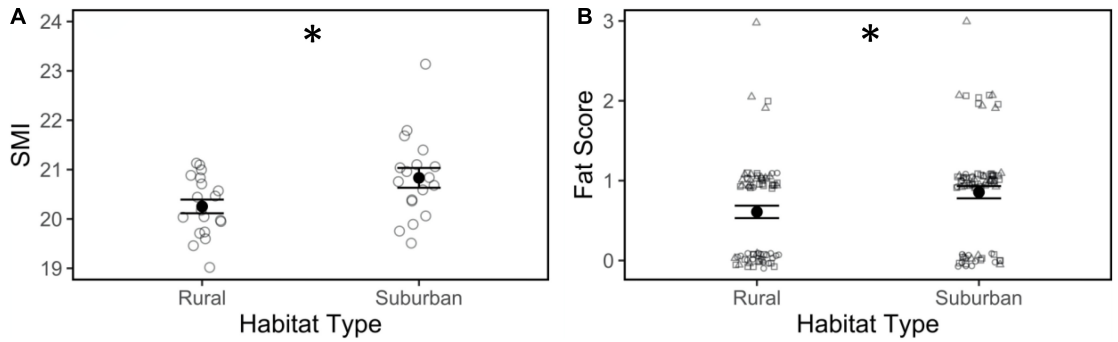

FIGURE 1 | Scaled mass index (SMI; **A**) and fat score **(B)** of male song sparrows from rural and suburban habitats. Closed circles and whiskers are mean ± SE; open shapes are measurements of individuals birds; **(B)** open squares: 2016; open circles: 2017; open triangles: 2020; asterisk denotes difference at $p < 0.05$.

TABLE 1 | Physiological measurements of male song sparrows collected from rural or suburban habitats.

	Rural (mean ± SE)	Suburban (mean ± SE)	Habitat:|*t*| value (*p*-value)	Time of day: |*t*| value (*p*-value)	Julian date:|*t*| value (*p*-value)	SMI: |*t*| value (*p*-value)
Condition indices						
SMI	20.25 ± 0.14	20.83 ± 0.20	**2.05 (0.048)**	0.51 (0.61)	0.09 (0.93)	n.a.
Fat score (range: 0–3)	0.61 ± 0.08	0.86 ± 0.08	**2.03 (0.04)**	1.82 (0.07)	0.68 (0.50)	1.12 (0.26)
Hematological indices						
PCV (%)	48.12 ± 0.66	46.48 ± 0.82	1.12 (0.27)	0.05 (0.96)	0.42 (0.68)	0.89 (0.38)
Hemoglobin (g/dL)	16.78 ± 0.26	15.86 ± 0.26	**2.96 (<0.01)**	1.16 (0.26)	1.02 (0.32)	0.18 (0.86)
MCHC (g/dL)	34.88 ± 0.23	33.77 ± 0.44	**2.96 (<0.01)**	1.82 (0.08)	0.98 (0.33)	1.08 (0.29)
Oxidative stress						
tGSH (μmol/mL)	4.13 ± 0.16	3.75 ± 0.15	0.84 (0.41)	0.45 (0.66)	0.70 (0.49)	1.42 (0.17)
GSSG (μmol/mL)	1.15 ± 0.05	1.06 ± 0.05	0.82 (0.42)	0.16 (0.88)	0.74 (0.46)	1.11 (0.28)
Free GSH (μmol/mL)	1.83 ± 0.9	1.64 ± 0.9	0.57 (0.58)	0.61 (0.55)	0.40 (0.69)	1.26 (0.22)
GSSG:GSH	0.63 ± 0.02	0.63 ± 0.03	0.02 (0.98)	0.18 (0.86)	0.48 (0.64)	0.13 (0.90)
dROM (mg H_20_2/dL)	12.86 ± 0.60	12.14 ± 1.20	1.24 (0.23)	0.40 (0.70)	0.84 (0.41)	0.57 (0.57)
Antioxidant Capacity (mM HOCL neutralized)	237.34 ± 8.72	243.98 ± 9.02	0.54 (0.59)	0.90 (0.37)	0.08 (0.94)	**2.27 (0.03)**
Immune function						
Heterophil (per 100 WBC)	22.11 ± 3.09	29.55 ± 2.87	**4.35 (<0.01)**	0.25 (0.80)	**2.45 (0.02)**	1.06 (0.29)
Lymphocyte (per 100 WBC)	74.06 ± 3.43	65.90 ± 3.01	**2.40 (0.02)**	0.29 (0.77)	**2.72 (<0.01)**	0.02 (0.98)
Monocyte (per 100 WBC)	3.83 ± 1.03	4.45 ± 0.87	1.37 (0.17)	0.95 (0.34)	**4.67 (<0.01)**	**2.98 (<0.01)**
H:L ratio	0.35 ± 0.06	0.44 ± 0.05	0.55 (0.59)	0.39 (0.70)	1.64 (0.11)	0.37 (0.72)
BKA (relative %)	58.85 ± 5.53	52.80 ± 4.09	0.71 (0.48)	0.13 (0.90)	**2.67 (0.01)**	0.79 (0.43)

Bolded values denote main effects at $p < 0.05$. PCV, packed cell volume; MCHC, mean corpuscular hemoglobin concentrations; tGSH, total glutathione; GSSG, oxidized glutathione; d-ROMs, reactive oxygen metabolites; HOCl, hypochlorous acid neutralization; H:L ratio, heterophil:lymphocyte ratio; BKA, bacteria killing ability.

$p = 0.047$), but fat score did not vary by body condition or time of day (**Table 1**).

Hematological Indices

Packed cell volume did not differ between suburban and rural birds (**Figure 2A**; $|t| = 1.12$, $p = 0.27$), but total hemoglobin was lower in suburban birds compared to rural birds (**Figure 2B**; $|t| = 2.96$, $p < 0.01$). Additionally, MCHC was lower in suburban birds compared to rural birds (**Figure 2C**; $|t| = 2.96$, $p < 0.01$). PCV, hemoglobin, and MCHC did not vary within the season, by time of day, or by body condition (**Table 1**).

Oxidative Stress

There was no effect of habitat on tGSH (**Figure 3A**; $|t| = 0.84$, $p = 0.41$), free GSH (**Figure 3B**; $|t| = 0.57$, $p = 0.58$), GSSG (**Figure 3C**; $|t| = 0.82$, $p = 0.42$), or GSSG:tGSH (**Figure 3D**; $|t| = 0.02$, $p = 0.98$), nor were these glutathione concentrations influenced by Julian date, time of day, or body condition (**Table 1**). Our measure of reactive oxygen metabolites, d-ROMs, did not differ between suburban and rural birds (**Figure 4A**; $|t| = 1.24$, $p = 0.23$), and did not vary within the season, by time of day, or by body condition (**Table 1**). Likewise, suburban and rural birds did not differ in antioxidant capacity (i.e., HOCl neutralization; **Figure 4B**; $|t| = 0.54$, $p = 0.59$). Antioxidant capacity was negatively related to body condition ($|t| = 2.27$, $p = 0.03$) but did not vary within the season or by time of day (**Table 1**).

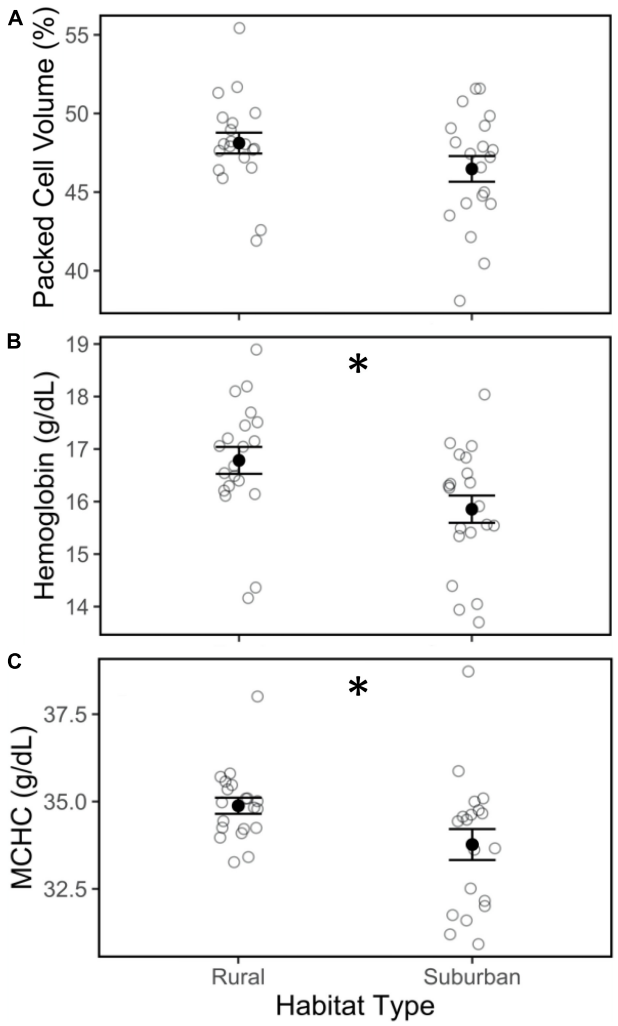

FIGURE 2 | Packed cell volume (PCV; **A**), total hemoglobin concentration **(B)**, and mean corpuscular hemoglobin concentration (MCHC; **C**) in blood collected from male song sparrows inhabiting rural and suburban sites. Closed circles and whiskers are mean ± SE; open circles are measurements of individuals birds; asterisk denotes difference at $p < 0.05$.

White Blood Cell Counts and Bacteria Killing Ability

Heterophil counts were higher in suburban birds compared to rural birds (**Figure 5A**; $|t| = 4.35$, $p < 0.01$). Conversely, suburban birds had lower lymphocyte counts compared to rural birds (**Figure 5B**; $|t| = 2.40$, $p = 0.02$). Monocyte counts did not differ between suburban and rural birds (**Figure 5C**; $|t| = 1.37$, $p = 0.17$). The abundance of heterophils ($|t| = 2.45$, $p = 0.02$) and monocytes ($|t| = 4.67$, $p < 0.01$) decreased as the breeding season progressed, whereas the abundance of lymphocytes ($|t| = 2.72$, $p < 0.01$) increased as the breeding season progressed. Additionally, there as a positive relationship between monocyte counts and body condition ($|t| = 2.98$, $p < 0.01$), but neither lymphocyte nor heterophil abundance varied by body condition (**Table 1**). White blood cell counts did not vary by time of day (**Table 1**). Although suburban birds exhibited a higher mean heterophil count and a lower mean lymphocyte count, there was no difference in H:L ratio between suburban and rural birds (**Figure 5D**; $|t| = 0.55$, $p = 0.59$), nor did H:L ratio vary within the season, by time of day, or by body condition (**Table 1**). We observed eosinophils in some cases, but overall abundance of this cell type was too low to compare across habitats.

Bacteria killing ability did not differ between rural and suburban birds (**Figure 6**; $|t| = 0.71$, $p = 0.48$) and did not vary by time of day or body condition (**Table 1**), but BKA did increase as the breeding season progressed ($|t| = 2.67$, $p = 0.01$).

Correlations Among Physiological Traits

We examined relationships among physiological traits for measurements collected in year 2020. Although we detected some significant correlations between variables that were experimentally derived from each other (e.g., mass and scaled mass index; tGSH and free GSH; MCHC and hemoglobin), there were no notable relationships among physiological variables (**Supplementary Table 2**).

DISCUSSION

Over the last 20 years, the field of urban ecology has burgeoned as the rate of urban expansion has accelerated around the globe (Hedblom and Murgui, 2017; Barot et al., 2019). However, even with increasing attention paid to interactions among wildlife and urban landscapes, it remains difficult to predict the costs and

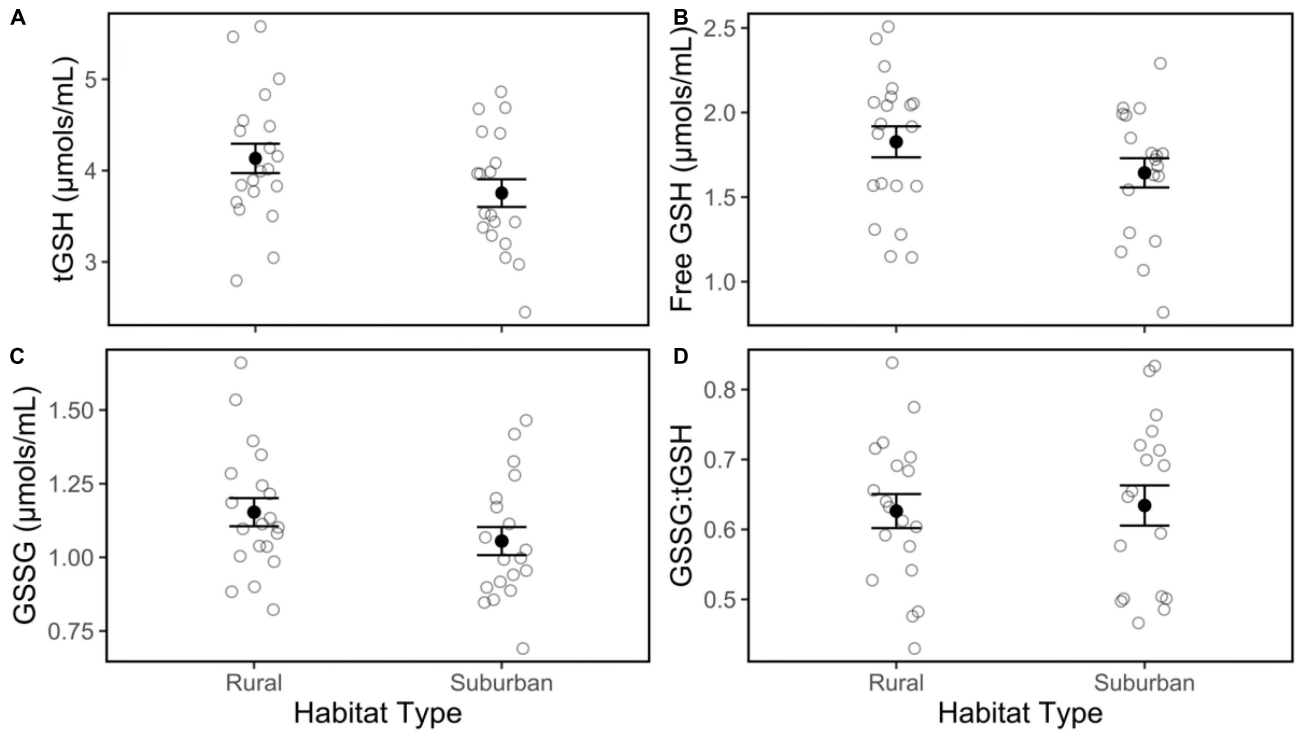

FIGURE 3 | Total glutathione (tGSH; **A**), free glutathione (GSH; **B**), oxidized glutathione (GSSG; **C**) concentrations and the ratio of GSSG:tGSH **(D)** in blood collected from male song sparrows inhabiting rural and suburban sites. Closed circles and whiskers are mean ± SE; open circles are measurements of individuals birds. No significant habitat differences were detected.

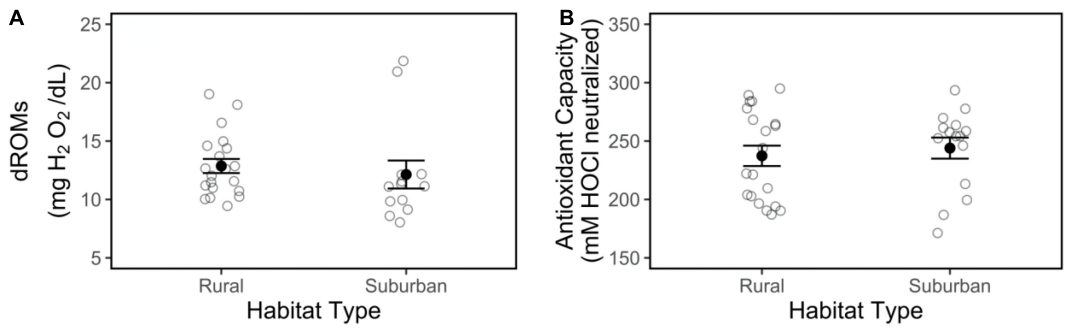

FIGURE 4 | Reactive oxygen metabolites (dROMs; **A**) and antioxidant capacity **(B)** in plasma collected from male song sparrows from rural and suburban sites. Closed circles and whiskers are mean ± SE; open circles are measurements of individuals birds. No significant habitat differences were detected.

benefits of urbanization for wildlife due to a lack of consensus on how animals respond physiologically to urbanization. Recently, there has been growing recognition that site-specific differences in biotic and abiotic variables are major factors determining how animals respond to urbanization (Isaksson, 2020). For example, the anthropogenic landscape of high-density metropolitan habitats differs dramatically from low-density suburban habitats (Chace and Walsh, 2006; Aronson et al., 2014). Though several studies have examined physiological responses in animals inhabiting high-density urban environments, comparatively few studies have examined physiological responses in wildlife inhabiting suburban environments.

In this study, we found that male song sparrows from rural and suburban habitats exhibited differences in physiological traits indicative of shifts in energy budgets. Specifically, song sparrows from the suburban habitat exhibited higher body condition and fat score but lower hemoglobin concentrations. We previously observed that song sparrows from these rural and suburban habitats exhibit behavioral differences (Beck et al., 2018; Davies et al., 2018) that may contribute to rural song sparrows experiencing a greater aerobic demand, thereby causing a depletion of fat reserves and an adaptive increase in hemoglobin concentration to facilitate oxygen delivery to tissues. Furthermore, given that we did not observe higher oxidative

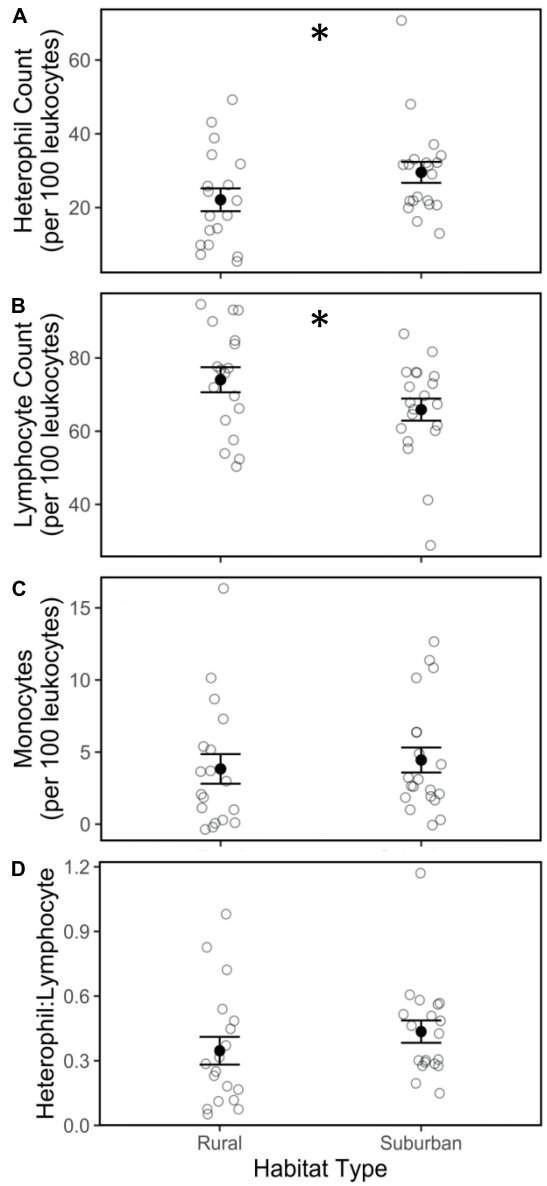

FIGURE 5 | Heterophil **(A)**, lymphocyte **(B)**, and monocyte **(C)** cell counts and heterophil: lymphocyte ratio **(D)** from male song sparrows from rural and suburban sites. Closed circles and whiskers are mean ± SE; open circles are measurements of individuals birds; asterisk denotes difference at $p < 0.05$.

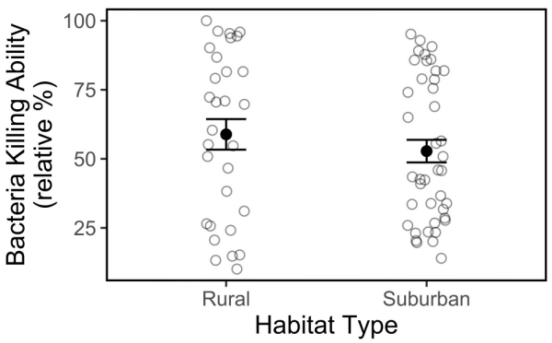

FIGURE 6 | Bacteria killing ability of plasma from male song sparrows from rural and suburban sites. Closed circles and whiskers are mean ± SE; open circles are measurements of individuals birds. No significant habitat differences were detected.

stress in suburban song sparrows, the lower hemoglobin concentration in suburban birds does not appear to be caused by oxidative damage to erythrocytes (discussed below). We also found some evidence that suburban and rural song sparrows differed in their white blood cell counts, but overall, low-density urbanization did not appear to substantially impact immune function (discussed below). Taken as a whole, the results from the present study (1) suggest suburban environments do not cause severe adverse physiological responses in song sparrows but may result in shifts in energy budgets, and (2) underscore the importance of measuring multiple physiological variables across sites that differ in the intensity of urbanization in order to avoid misinterpreting the impact of urbanization on avian health.

Effects of Low-Density Urbanization on Oxidative Stress, Body Condition, and Hemoglobin

The finding that suburban song sparrows exhibited lower hemoglobin concentrations compared to rural counterparts is consistent with previous studies that examined hemoglobin concentration in noisy miners (*Manorina melanocephala*) and house sparrows (*Passer domesticus*) from rural and urban habitats (Herrera-Duenas et al., 2014; Powell et al., 2014; but see Minias, 2016). Exposure to urban contaminants and anthropogenic noise can increase oxidative stress, resulting in urban birds exhibiting oxidative damage to red blood cells and lower PCV and hemoglobin concentrations in urban birds (Herrera-Duenas et al., 2014; Bauerová et al., 2017). However, unlike these previous findings, we did not detect differences in GSH concentrations, d-ROMs, or antioxidant capacity (HOCl neutralization) in song sparrows at our suburban sites. Therefore, the cause for this difference in hemoglobin concentrations may not be a direct effect of urbanization as it is in prior studies.

There are two possible explanations for the lack of habitat-specific differences in oxidative stress. It may be that song sparrows are excellent urban adapters, whereby urbanization does not substantially alter oxidative status. Alternatively, it is possible that song sparrows can cope with low-density urbanization but exposure to higher intensity urbanization of large metropolitan habitats would increase oxidative status. Collecting song sparrows from high density urban habitats would aid in the ability to interpret the effect of low-density suburban habitats on song sparrows; however, due to the absence of high density urbanization on the landscape of our study system, we were unable to measure physiological variables in song sparrows from highly urbanized environments.

Similar to our results, Herrera-Dueñas et al. (2017) found minimal evidence of oxidative stress in house sparrows inhabiting suburban habitats, though these authors did observe higher

oxidative stress in house sparrows from highly urbanized habitats compared to birds from rural habitats. Given birds inhabiting suburban environments are less likely to be exposed to contaminants (e.g., nitrogen oxides, heavy metals, and pesticides) found at higher concentrations in high-density urban environments (e.g., Espín et al., 2014; Salmón et al., 2018), it is unsurprising that we did not detect higher oxidative stress in suburban song sparrows in our study. Yet, the absence of oxidative stress in song sparrows from our study system also rules out the possibility that damaging effects of oxidative stress are causing a reduction in hemoglobin concentrations in male song sparrows from the suburban habitat.

Though it is difficult to identify the specific environmental or ecological factors driving differences in hemoglobin concentrations among rural and suburban song sparrows, the overall pattern of physiological differences suggests an indirect effect of urbanization on energy budgets. Specifically, in addition to higher hemoglobin concentrations, rural song sparrows also exhibited lower body condition (i.e., scaled mass index) and fat scores. These findings suggest that rural birds may experience a greater metabolic demand than their suburban counterparts and that these differences reflect adaptive physiological responses to environmental variation.

Our previous work in this song sparrow system suggests habitat-specific differences in behavior may contribute to rural birds allocating more energy toward activity. For instance, suburban song sparrows are more aggressive than their rural counterparts when exposed to simulated conspecific territorial intrusions conducted with taxidermic mounts (Davies and Sewall, 2016; Beck et al., 2018; Davies et al., 2018). Interestingly, although suburban song sparrows exhibit a higher aggression score in any single interaction, conspecific density is nearly ninefold greater in the rural song sparrow population (unpublished data), and higher neighbor density can increase the frequency of aggressive interactions in territorial songbirds (Yoon et al., 2012), even while decreasing the intensity of those conflicts (the so-called "Dear Enemy" effect, Fisher, 1954; Temeles, 1994). Therefore, even though rural song sparrows are less aggressive in a given interaction, rural song sparrows likely allocate more energy toward frequently defending their territories from conspecific intrusions compared to suburban song sparrows. Furthermore, based on personal observation (KBS) over 8 years of working with this song sparrow population, rural birds typically take longer to respond to conspecific playbacks than suburban males, and we often attract multiple rural males during a single playback, whereas we rarely encounter multiple males on territories of suburban sparrows. These anecdotal observations suggest rural song sparrows are more likely to travel further from their territory. If rural song sparrows must defend their territories more frequently and tend to travel further from their territory than suburban song sparrows, it is likely that rural birds experience a higher aerobic demand for greater activity.

Other behavioral differences between urban and rural birds may also be associated with rural birds experiencing a greater aerobic demand. Several studies, including a recent study in our lab (unpublished data), have found that rural song sparrows have a longer flight initiation distance (i.e., birds tended to fly away when observers were further way) compared to suburban birds (Evans et al., 2010; Fossett and Hyman, 2021), a response that has been observed in other avian species as well (Lin et al., 2012; Møller et al., 2013; Carlen et al., 2021). Flight initiation distance, in turn, is positively correlated with basal metabolic rate (BMR), suggesting BMR may be higher in rural birds as a consequence of longer flight initiation distance and the frequency of such flights (Møller, 2009a). Considering the behavioral differences in our song sparrow population as well as potential associations between habitat-specific differences in flight initiation distance and BMR, it is possible that rural birds are adaptively increasing hemoglobin concentrations to facilitate greater aerobic scope. Such an increase is consistent with previous studies that report birds adaptively adjust hemoglobin concentrations to meet energetically demanding situations (e.g., Jaeger and McGrath, 1974; Prats et al., 1996; Bury et al., 2019). Although several studies have examined hematological indices and oxidative stress in urban and rural populations, fewer studies have quantified whether urban and rural birds have distinct metabolic phenotypes. Future research should examine whether habitat-specific differences in hemoglobin concentrations translate to differences in metabolic rates to determine if the differences we observed in hemoglobin concentrations indeed reflect shifts in aerobic demand.

Effects of Low-Density Urbanization on Immune Function

Song sparrows from suburban habitats exhibited slightly elevated heterophil counts compared to their rural counterparts, though the biological significance of this finding is somewhat unclear. Several previous studies have found birds inhabiting urban environments experience increased risk of infection from avian diseases (e.g., avian malaria, poxvirus, and coccidia) compared to rural dwelling birds (Bichet et al., 2013; Jiménez-Peñuela et al., 2019; Sykes et al., 2021). Consequently, birds inhabiting high-density urban environments tend to have greater investment in innate immunity (Møller, 2009b; Audet et al., 2016). Despite this evidence, the incidence and severity of infection in birds tends to be positively correlated with the intensity of urbanization, suggesting both infection and immune activation will be highest in large cities (Giraudeau et al., 2014). Because the suburban sites in our study were characterized by low-intensity urbanization, it is unlikely that suburban song sparrows experience greater disease exposure, which may explain why we did not observe a substantial difference in heterophil counts between rural and suburban song sparrows.

Although male song sparrows in our study exhibited higher heterophil counts, there was no difference in BKA or H:L ratio, which is a measure of innate immune response that is closely associated with the long-term activation of the corticosterone-mediated stress response in birds (Davis et al., 2008; Minias, 2019). Given that we did not observe differences in BKA or H:L ratios, the suburban sites do not appear to induce a readiness to cope with infection or a state of chronic stress in male song sparrows. This finding is generally consistent with results of our

previous studies, where we did not detect differences in baseline or stress-induced plasma corticosterone levels in song sparrows from rural and suburban habitats (Beck et al., 2018; Davies et al., 2018). Additionally, there was no difference between suburban and rural males in their ability to terminate the stress response following dexamethasone injection (Lane et al., 2021). While some other studies have detected differences in corticosterone levels between rural and urban dwelling birds (e.g., Bonier et al., 2007; Ibáñez-Álamo et al., 2020), overall there does not appear to be a consistent relationship between urbanization and the corticosterone-mediated stress response in birds (Bonier, 2012; Injaian et al., 2020).

CONCLUSION

We observed that suburban birds exhibited higher body condition and fat scores but lower hemoglobin concentrations, indicating song sparrows adaptively respond to habitat-specific aerobic demands by altering hemoglobin concentration. To test the hypothesis that habitat differences shift energy budgets in song sparrows, future research should focus on quantifying differences in aerobic performance among rural and suburban song sparrows. For example, future work should examine whether variation in hemoglobin concentrations among rural and suburban sparrows is associated with resting and maximum metabolic rates. Additionally, hemoglobin synthesis can be suppressed by poor nutrition (Minias, 2015), so future studies should also examine whether availability of micronutrients on the landscape influences the metabolic phenotypes of rural and suburban song sparrows (Coogan et al., 2018; Cummings et al., 2020a). Finally, to better understand the ecological relevance of varying hemoglobin levels, it is critical to understand whether hemoglobin concentration is related to breeding ecology and fitness outcomes (e.g., survival and reproductive success). We note that characterization of urban environments and the use of "urban" vs. "suburban" vary among studies, which limits the ability to perform comparative analyses. Therefore, greater emphasis should be placed on employing similar methodologies for characterizing urban environments among studies in order to facilitate comparisons of phenotypic responses to urbanized habitats. In the context of the present study, low-intensity urbanization does not appear to cause severe adverse physiological responses in song sparrows, rather physiological differences among rural and suburban sparrows appear to be adaptive to habitat specific biotic and abiotic factors.

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 reviewed and approved by the Virginia Tech IACUC.

AUTHOR CONTRIBUTIONS

CG and KS designed the study. SL, IV, MB, and HE contributed to data collection. CG and SL contributed statistical analysis. CG, KS, and IV wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.817864/full#supplementary-material

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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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Diluted Seawater and Ammonia-N Tolerance of Two Mangrove Crab Species. New Insights to Understand the Vulnerability of Pristine Islands Ecosystems Organisms

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Mangrove ecosystems are the primary receptors of anthropogenic pollution in tropical areas. Assessing the vulnerability of these ecosystems can be expressed, among other indicators, by studying the health of 'ecosystem engineers'. In this study, mangrove forests facing opposing anthropogenic pressures were studied (i) in the uninhabited island of Europa (Mozambique Channel), considered as a pristine ecosystem, and, (ii) on the island of Mayotte, facing regular domestic wastewater discharges. Using an ecophysiological approach, the effects of diluted seawater (DSW) and increased ammonia-N were studied for two fiddler crab species: *Gelasimus tetragonon* (GT) on the island of Europa and *Paraleptuca chlorophthalmus* (PC) on the island of Mayotte. Osmoregulation curves and osmoregulatory capacity were determined along with O_2 consumption rates after a 96 h exposure period. Histological analyses were also carried out on two important metabolic organs: the hepatopancreas and the posterior gills. Results indicate that both crab species are good hyper-hypo-osmoregulators but only PC can maintain its osmoregulatory capacity when exposed to ammonia-N. Oxygen consumption is increased in GT after 96 h of exposure to ammonia-N but this does not occur in PC. Finally, a thickening of the gill osmoregulatory epithelium was observed after 96 h in PC when exposed to ammonium but not in GT. Therefore, the two species do not have the same tolerance to DSW and increased ammonia-N. PC shows physiological acclimation capacities in order to better manage nitrogenous enrichments. GT did not show the same physiological plasticity when exposed to ammonia-N and could be more at risk by this kind of stress. These results along with those from other studies regarding the effects of domestic effluents on mangrove crabs are discussed. Therefore, the greater vulnerability of organisms occupying pristine ecosystems could induce major changes in mangrove functioning if crabs, that are engineer species of the ecosystem, are about to reduce their bioturbation activity or, even, disappear from the mangrove forests.

Keywords: Crustacea, pristine ecosystems, biomarkers, metabolism, ammonia-N, mangrove

INTRODUCTION

Ecological and ecosystem vulnerability assessment may represent a valuable tool in biodiversity risk management (De Lange et al., 2010). From the definition of Ippolito et al. (2010), ecosystem vulnerability may represent "the potential of an ecosystem to modulate its response to stressors over time and space, where that potential is determined by characteristics of an ecosystem vulnerability that include many levels of organization." Ecosystem vulnerability assessment may be expressed with a level of potential impact related to a certain stressor in a given environment: "the actual status of a polluted ecosystem or community represents the response of a (more or less) pristine ecosystem or community to a specific stressor or to multiple stressors" (De Lange et al., 2010). The authors, thus, state that combining the assessment of vulnerability of a pristine ecosystem or community with the actual status allows obtaining crucial information for risk management.

Among vulnerable ecosystems, mangrove forests that thrive intertidal areas are typically subjected to a variety of stressors, both natural and anthropogenic. These include high levels of pollutants and nutrients, severe hypoxia, high water turbidity, and fluctuations in temperature and salinity. Mangroves are significant ecosystems to study given their ecological importance in tropical areas. They accomplish a wide range of key ecosystem functions such as carbon storage, nutrient and global carbon cycling, provide breeding, recruitment, nursery and feeding areas both terrestrial and aquatic fauna (Nagelkerken et al., 2008; Amaral et al., 2009; Cannicci et al., 2009; Lugendo and Kimirei, 2021). Also, mangrove forests form a buffer zone protecting the shoreline against erosion and watershed (Zhang et al., 2012; Ouyang and Guo, 2016).

Located at the land-sea interface, mangroves are the receptacle of anthropogenic activities such as aquaculture, agriculture, urbanization, tourism or coastal pollution (Thomas et al., 1996; Alongi, 2002; Xiang et al., 2020; Lugendo and Kimirei, 2021).

Nitrogen inputs and the altered global cycle of N are one of the most important concerns about anthropogenic presence and activities (Vitousek et al., 1997). Human alterations of the nitrogen cycle can lead, amongst others, to changes in the composition and functioning of estuarine and nearshore systems, with accelerated losses of biological diversity (Nixon, 1995; Nixon et al., 1996; Vitousek et al., 1997). It has been predicted that tropical regions will receive the most dramatic increases in reactive N (Nr) inputs over the next few decades (Zhu et al., 2005; Galloway et al., 2008). In this context, our study aims at comparing some features of ecosystem vulnerability in both pristine and anthropized mangrove ecosystems.

Europa island (22° 20′ S, 40° 22′ E) is the southernmost island of the Mozambique Channel. It is considered a pristine ecosystem as there is no permanent human settlement, making it a prime candidate for marine conservation (O'Donnell et al., 2017). Europa island is a low-lying (7 m elevation), coral island of 28 km^2 surrounded by sand dunes. A shallow lagoon, almost empty at low tide, is located on the north-western part of the island. It is fringed with 626 ha of mangrove forest (Boullet, 2014; Juhel et al., 2019).

Mayotte island (12°50′S 45°08′E) is a tropical island (374 km^2) situated in the Comoros Archipelago, in the northern part of the Mozambique Channel. On this island, the mangrove forest covers about 700 ha of the territory (Jeanson et al., 2019). Large ecological gradients are encountered in Mayotte rivers and mangroves, one of the main anthropogenic pressures being the lack of connection between households and the sewage systems. Therefore, domestic wastewater is often discharged directly into rivers and mangroves (Herteman, 2010; Vasselon et al., 2017; Capdeville, 2018). Furthermore, the island of Mayotte possesses a fast growth of its human population, especially over the last two decades resulting in an exponential increase in anthropogenic pressures and degradations of its marine environment (Pusineri et al., 2014).

Studying ecosystem vulnerability in response to stressors must take into consideration indicators of ecosystem functioning, including early warning, diagnostic and retrospective indicators. Aquatic organisms including crustacean species, are frequently studied as early warning indicators of changes in environmental conditions (Burger, 2006; Ungherese and Ugolini, 2009). Some ecological functions provided by these species, such as ecosystem engineering, have also been identified (Brodie et al., 2018) as key elements to incorporate into conservation planning (see Coggan et al., 2018 for a review). Ecosystem engineers are species whose actions have "significant impacts" upon the physical structure of their habitat and the organisms that live in them (Jones et al., 1994). Amongst them, we find burrowing mangrove fiddler crabs, considered as ecosystem engineers through their bioturbation activity (Kristensen, 2008; Penha-Lopes et al., 2009; Kristensen et al., 2012). Their activity briefly consists in burying, macerating and ingesting litter as well as actively digging and maintaining burrows in the sediment (Giddins et al., 1986; Emmerson and McGwynne, 1992; Dittmann, 1996; Lee, 1997; Kristensen and Alongi, 2006).

We chose to study two ecologically, morphologically and phylogenetically proximate species of Ocypodids fiddler crabs: *Paraleptuca chlorophthalmus* (PC) from the island of Mayotte and *Gelasimus tetragonon* (GT) from the island of Europa (**Figure 1**). As fiddler crabs, these two species are mainly characterized by a sexual dimorphism where female possess two small feeding claws when males possess one enlarged claw (up to 50% of total bodyweight). PC distribution range is restricted to the East African province of the Indian Ocean Subrealm of which it is endemic, whereas GT is the fiddler crab with the largest distribution range. It is widespread in the Indian Ocean and also in the Pacific Ocean (**Figure 1**) (Rosenberg, 2020). Only one observation of GT has been reported in Mayotte Island (Bouchard et al., 2013). PC has also been observed once in Europa Island (Poupin et al., 2012). We did not confirm this observation and the DNA sequencing analysis performed on the crabs collected on Europa Island did not reveal any representative of this species (see **Supplementary Material**). These two species are the most abundant crab species living in semi-open canopy habitats situated near tidal rivulets of the two studied mangroves. In these two areas, a high density of burrows (that originates from bioturbation activity) is observed. These habitats are the first receptors of anthropogenic spillages in the island of Mayotte.

Salinity variations throughout freshwater inputs and high levels of nutrients are two key stressors in such context. On the island of Europa, rainfall is the only source of freshwater, with a mean annual rainfall of 540 mm/year and a long dry period of 7 months in a semi-arid climate (Boullet, 2014; Lambs et al., 2016). Therefore, GT lives under strong marine influence, mostly in seawater and oligotrophic conditions. On the island of Mayotte, 24 permanent rivers reaching the sea have been listed (Lapègue, 1999), with a watershed area ranging from 2 to 23 km^2 (Lagarde et al., 2021). Therefore, PC is frequently experiencing salinity variations and osmotic changes under mesotrophic or eutrophic conditions, especially in anthropized areas.

In habitats with frequently changing environmental conditions (such as mangrove forests), organisms are exposed to multiple stressors that can neutrally, synergistically or antagonistically interact (Piggott et al., 2015; Renault et al., 2018). In order to maintain functional homeostasis, phenotypic adjustments may be expressed during the life history of an individual, ranging from physiological adjustments (acclimation) (Teets and Denlinger, 2013) to phenological changes and range shifts (Chuine, 2010; Briscoe et al., 2012; Renault et al., 2018). In some cases, these exogenous factors can generate physiological evolutionary changes because of genetic modifications (adaptation). This study does not aim to determine any potential environmental adaptation in the event that different sensitivities are observed between crab populations, and this should remain a working hypothesis only.

Considering these elements, we chose to study physiological endpoints on different levels of biological organization to assess the potential effects of salinity variations and short-term exposures to ammonia-N on the two crab species. Physiological biomarkers such as osmoregulatory capacity (the difference between hemolymph osmotic pressure and that of the external medium, OC), oxygen consumption and histological changes have been widely studied and proved to be informative proxies of crustacean responses to environmental changes such as salinity variations and ammonia-N inputs (Lignot et al., 2000; Gillikin et al., 2003; Ortega et al., 2017; Theuerkauff et al., 2018a,b; Ros et al., 2021). Resting metabolic rate is also considered as physiologically relevant (Ikeda, 2016; Borges et al., 2018) to assess the potential effects of an osmotic chock or ammonia-N exposure on crabs, independently from a potential locomotor or escape behavior.

Therefore, could GT and PC show different physiological tolerances to basic anthropogenic pressures, here: salinity reduction and high ammonia-N inputs? The working hypothesis is that vulnerability to diluted seawater and ammonia-N inputs must be different for the two species. Specifically: (1) PC could have a good tolerance capacity to reduced salinity and increased ammonium input within a short-term acclimation period; (2) GT, that is mainly recorded in stable environments, i.e., rarely exposed to eutrophic water and rarely to diluted seawater (Crane, 1975; Weis and Weis, 2004), could be vulnerable to anthropogenic discharges inducing salinity decrease; (3) there should be deleterious effects of Ammonia-N on osmoregulatory processes (aggravating factor) for both species since ammonia-N is known to disrupt physiological processes such as osmoregulation, immunology, acid/base balance and gas exchange in Decapod crustaceans (Weihrauch et al., 2004; Romano and Zeng, 2013).

In the present study, crabs were collected from Europa (GT) and Mayotte (PC) mangroves and were artificially exposed to diluted seawater (5 ppt) and Ammonia-N (10 $mg.L^{-1}$), a proxy of anthropogenic presence through an environmental concentration. Several biomarkers were considered in order to: (i) define the salinity tolerance range of the two species (osmolality curves), (ii) understand whether eutrophication can have an impact on the crab ability to maintain its hydromineral balance (osmoregulatory capacity), and, (iii) determine whether short-term acclimation (oxygen consumption and histological analyses) can occur to cope with eutrophication. If GT was found to be vulnerable to freshwater and Ammonia-N, what would be the consequences in terms of vulnerability to anthropogenic pressure for the pristine mangroves of the island of Europa? What could the consequences be in terms of risk assessment?

MATERIALS AND METHODS

Study Sites

Europa Island

The sampling site is mainly constituted of shrubby *Rhizophora mucronata* on coral mud with a sandy-silty texture, in an almost open canopy (**Figure 2**), near tidal channels and rivulets. The absence of freshwater inflows induces no transfer of nutrient or ions from inland to the mangrove (Lambs et al., 2016). High concentrations of nitrogen and phosphorus can be found in this Island on seabirds nesting sites due to amounts of guano, but the chosen sites in this study were not concerned (Zubia et al., 2016).

Mayotte Island

The study site is located in Boueni bay (12°55′1″S, 45°9′23″E) in the mangrove of Malamani, extending along both sides of a freshwater stream. On this site, vegetation structure is mainly represented by *Ceriops tagal* and *R. mucronata facies*, along with *A. marina* and *Bruguiera gymnorhiza* species. This open habitat presents no canopy cover, a large temperature gradient and regular water immersion/emersion, depending on the season, tides and time of the day. Malamani is part of the Chirongui area in the bay of Boueni, which had a human population density of 310 hab./km^2 in 2017.

A total of 30 crabs (15 from Europa and 15 from Mayotte) used during experiments were randomly chosen for DNA sequencing to confirm their taxonomical identification through a classical barcoding process (see **Supplementary Material**).

Animal Collection and Acclimation

Male and female GT and PC (the same number for each sex) were collected in April 2019 and March 2020, respectively, in the mangroves of Europa and Mayotte (Malamani area) (see **Figure 2** for the sampling site). All animals were hand collected at low tide when crabs are active and out of their burrows. They were placed into individual bags or boxes in order to minimize stress and fights during transport to the laboratory

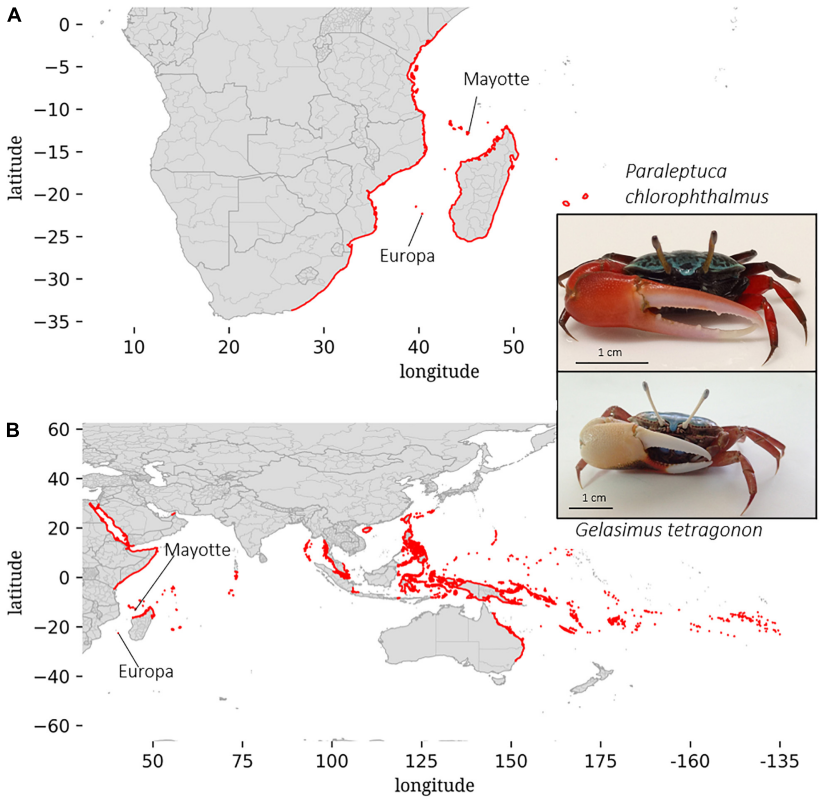

FIGURE 1 | Geographical ranges (in red) and pictures of the two studied species: *Paraleptuca chlorophthalmus* **(A)** and *Gelasimus tetragonon* **(B)**. Data was generated from the cybertaxonomy database described in Rosenberg (2014) and from the website: www.fiddlercrab.info.

(either the field laboratory on Europa Island, or the CUFR marine laboratory at the University Center of Mayotte). Animals (∼130 for PC, ∼100 for GT) were placed for 3 days for acclimation in individual boxes containing natural filtered seawater (∼33‰ salinity; 1,050 mOsm.kg^{-1}), under a natural photoperiod (12 h light: 12 h dark) and without feeding prior to experimentation.

For the osmoregulation experiment, ∼100 PC were studied (N = 10 per salinity) in Mayotte and ∼70 GT were studied (N = 4–8 per salinity) in Europa Island. Sampling size was limited here due to the pristine nature of the ecosystem and legal environmental protection requirements. For the respirometry and osmoregulatory capacity, 24 crabs were studied for each species. No mortality was observed during acclimation and experimental processes for PC (Mayotte). No mortality was observed during acclimation for GT (Europa), but one female died after 72 h of ammonia-N exposure.

Ammonia-N Exposure and Experimental Conditions

Three different experimental conditions were performed: some crabs were maintained in seawater (∼33 ppt), others were transferred to diluted seawater (∼5 ppt) or to 10 mg.l^{-1} ammonia-N solution (hereafter noted, respectively, SW, DSW and N exposure). This solution was prepared by adding 1 mMol of ammonium chloride (Sigma, United States) to diluted seawater (∼5 ppt). Ammonia-N was chosen as experimental treatment because the ionized form of ammonia-N ($NH4^+$) is the major component of domestic WW (Capdeville et al., 2018; Mégevand et al., 2021, submitted). This condition refers to total ammonia (ammonia-N). It represents the sum of unionized ammonia NH3 and ionized ammonia $NH4^+$ (Haywood, 1983; Lemarié et al., 2004; Bermudes and Ritar, 2008). The ammonia-N concentration was chosen on the basis of concentrations that may be released by treatment plants in Mayotte (Herteman, 2010). It corresponds to a sub-lethal concentration for a fiddler crab species such as *Uromastyx princeps*, adults sharing similar size and morphology with *P. chlorophthalmus* (Azpeitia et al., 2013). Ammonia-N exposure solutions were replaced every day. Salinity of SW and diluted seawater (DSW) were tested every day and readjusted if needed.

Osmolality Curves

To determine osmolality curves, crabs were exposed at different salinities with 4–8 crabs per salinity for GT, and 8–10 crabs per salinity for PC. Salinity ranged from DSW (∼4.5 ppt, ∼135 mOsm.kg^{-1}) to concentrated SW (∼51 ppt, ∼1,510 mOsm.kg^{-1}). Animals were maintained in isolated boxes for a minimum of 72 h, which is sufficient time for osmoregulation to be achieved in such estuarine crustacean species (Lovett et al., 2001; Rivera-Ingraham et al., 2016).

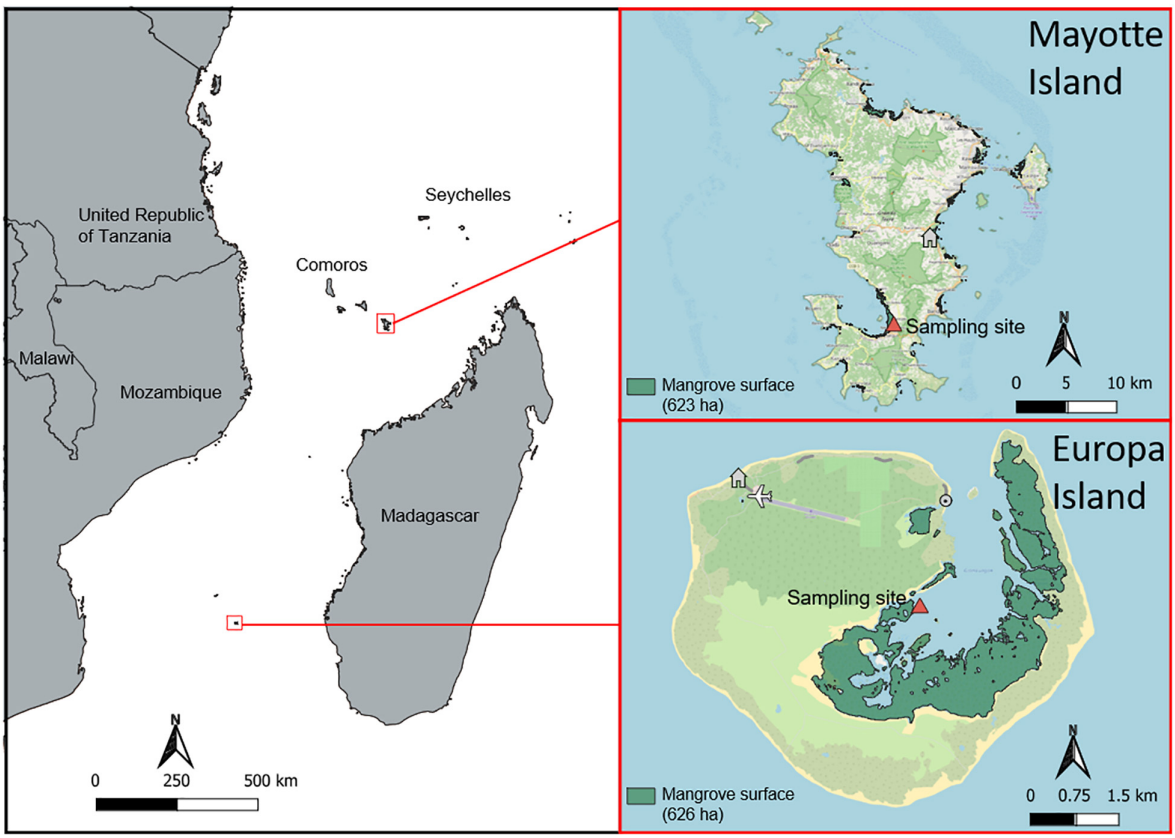

FIGURE 2 | Localization of Mayotte Island and Europa Island in the Mozambique Channel with the sampling sites.

After this period, a hemolymph sample was taken using a 0.1–0.5 ml hypodermic syringe with the needle inserted between the cephalothorax and the third and fourth periopod. Osmotic pressure (OP) of the hemolymph samples was immediately quantified in duplicate by freezing point depression osmometry (Model 3320, Advanced Instruments, Inc., Norwood, MA, United States).

At the end of this experiment, animals were put back in individual boxes in aerated SW before being released into the mangrove.

Oxygen Consumption Rate

The experimental design consisted in a static, intermittent flow-through respirometry system based on Clark et al. (2013). Crabs were individually placed into 125 ml chambers allowing them to make sporadic movements considering their size, while ensuring accurate measurements of O_2 consumption. Following procedures described in Killen (2014), O_2 measurements were performed using a 4-channel fiber-optic system with contactless O_2 sensor spots (FireSting O_2, PyroScience, GmbH, Aachen, Germany) where water oxygen content was quantified once every 5 s.

Water-mixing within the chambers was achieved with magnetic stirrers located under 1 mm^2 mesh grid to avoid too much disturbance (Rivera-Ingraham et al., 2016). Sensors were calibrated to 100 and 0% air saturation using air-saturated water and 80 mM Na_2SO_3 solution, respectively. Chambers were filled with a tubing system providing control SW, DSW or contaminated water from aerated, filtered and temperature-controlled tanks (∼25°C). Crabs were gently introduced in the chambers and left to acclimate for 1 h in aerated seawater prior experiment.

Following acclimation, the system was filled with the exposure solution. A flush pump was switched on for 20 min to ensure proper mixing and was cut off during 40 min. During that time, the chambers were sealed. The decrease in oxygen content could be analyzed to indicate the rate of oxygen uptake. After the 40-min cycle, the pump was turned on to flush the metabolic chambers with aerated seawater (or DSW or DSW enriched with ammonia-N) during 20 min. Due to the presence of aerobic and anaerobic microorganisms capable of degrading organic compounds and consuming O_2 (Shchegolkova et al., 2016), a 20 min flushing was run to ensure 99% air saturation. The 40 min measurements allowed to make accurate O_2 measurements without falling under the threshold of 70% air saturation in order to maintain aerobic metabolism and to avoid hypoxic stress (Ombres et al., 2011; Rodgers et al., 2016). O_2 measurements were taken at T-1h (SW), T1h, T24h, and T96h after exposure (SW as control treatment, DWS or DSW with ammonia-N) for all individuals (N = 8 per condition). This 96 h exposure time

was chosen in order to better compare our results with data from the literature focused on the effects of pollutants and particularly ammonia-N on crustaceans. Many studies are based on the 96h LC50 with environmental concentrations of ammonia-N and regularly include histological analyses (De Freitas Rebelo et al., 2000; Wang et al., 2003; Barbieri et al., 2016; Weihrauch and O'Donnell, 2017). Only one female *GT* exposed to ammonia-N died after 72 h of exposure.

Osmoregulatory Capacity

At the end of the 96 h respirometry exposure, a hemolymph sample from each animal was taken (N = 7–8 per condition) and OP was assessed as described above.

Sampling and Dissections

Following the 96 h respirometry experiment, crabs were anesthetized and euthanized on ice for tissue sampling of the hepatopancreas, anterior and posterior gills in order to perform the histology measurements. Samples were fixed in Bouin's fixative solution for 48 h for histological analyses. Individual molting stage was verified through examination of an epipodite under a dissecting microscope after dissection (to avoid handling-associated stress on the biological markers considered).

Histological and SEM Analysis

For histological analyses, posterior gills (pair 7 or 8) and small samples of the hepatopancreas were rinsed in 70% ethanol following immersion in Bouin's fixative solution for 48 h. They were then dehydrated in a series of graded alcohols and embedded in paraffin. Tissue sections (4 μm) were cut with a Microtome 2125RT Leica, then placed on Glycerin albumin precoated glass slides and deparaffinated. They were stained following Masson's Trichrome Staining Protocol (Martoja and Martoja-Pierson, 1967) using Haematoxylin Groat, Fuschine Ponceau and Aniline Blue staining solutions. Samples were examined using Leica DM6 microscope equipped with a Leica DMC 2900 and the associated software LAS X.

For the hepatopancreas analyses, pictures of the hepatopancreas for each individual were taken, each of them depicting 1–4 tubules. Images were scaled and analyzed using ImageJ software (version 1.51r). Tubules of the hepatopancreas were randomly selected for each individual. Four parameters were measured on each of those hepatopancreas tubules and, then, averaged for each individual (N = 3–8): the area of the tubules, the number of vacuoles per tubule, the area of the B-cell vacuoles, the percentage of B-cell vacuolization area per tubule. Posterior gill epithelial thicknesses were measured from areas where nuclei were visible using a 40× objective (**Figure 6C**). Along this single-layered epithelium, cell heights were systematically measured from the base of the cuticle to the basal side of the cells facing the hemolymph lacuna. For statistical purposes, gills were divided into three sections along longitudinal (basal, central, and apical) and transversal axes (proximal, middle, and distal). For each of these areas, photographs were taken at the same magnification (60×) using the CapturePro software. Measurements of epithelial gill thickness were then performed using Image as well.

STATISTICAL ANALYSIS

Statistical analyses were performed in R version 3.5.2 with Rstudio version 0.99.491 (Rstudio, Inc.), with an α-level of 0.05 and 95% confidence intervals used to determine statistical significance in all tests. For osmoregulatory capacity and gills epithelial thickness, residuals were evaluated using Shapiro–Wilk and Levene's test, respectively, in order to test the data normal distribution behavior and variance homogeneity. One-way ANOVAs were performed separately for posterior gills at each longitudinal level (apical, central, and basal) to assess potential differences of epithelial thickness between treatments (SW, DSW, and N) after 96 h exposure. To test potential effects of treatment on hepatopancreas parameters, we used generalized mixed-effects modeling with treatment as random effects (Manning, 2007; Zuur et al., 2013; Johnson et al., 2015) using the package glmmTMB (Brooks et al., 2017). The number of vacuoles per tubule, the mean area of the B-cell vacuoles per tubule, the percentage of B-cell vacuolization area per tubule in hepatopancreas were modeled in function of treatment (three-levels fixed factor) assuming a Gaussian distribution error with log link. Individual differences and repeated measurements among crabs and tubules were taken into account through a random intercept of tubule ID nested into crab ID. Then, type II ANOVAs (Wald chi-square test) were applied on the models using Anova function in CAR package (Fox and Weisberg, 2019). When significant effects of treatment were detected, they were investigated through pairwise comparisons using lsmeans package (Lenth, 2016) with Tukey correction. For O_2 oxygen consumption, a generalized linear mixed model approach was taken assuming Gaussian distribution error with log link. Time (T-1h, T0h, T24h, and T96h) and treatment group (SW, DSW, and N) were considered as fixed factors. Crab ID was set as random factor nested within experimental tank (two tanks of exposure solution per treatment, N = 4 per tank), which are nested within treatment group (SW, DSW, and N). Interaction terms were examined using the approach using type II ANOVA and multiple pairwise comparisons followed by Bonferroni correction. All values are represented as average ± SEM.

RESULTS

Tolerance to Salinity Variations and Osmoregulatory Capacity Under Osmotic and Ammonia-N Stress

Both GT and PC are strong osmoregulators across the tested salinities since they can maintain a gradient of greater than 600 $mOsm.kg^{-1}$ above the ambient medium when exposed to salinities close to FW (5–10 $mOsm.kg^{-1}$, **Figure 3A**). Both species hyper-regulated at lower salinities (hemolymph osmolality actively maintained at levels above that of DSW) and hypo-regulated at higher salinity (hemolymph osmolality maintained below that of SW). The difference between external medium and hemolymph osmolality significantly increased as salinity decreased when both species were exposed to DSW

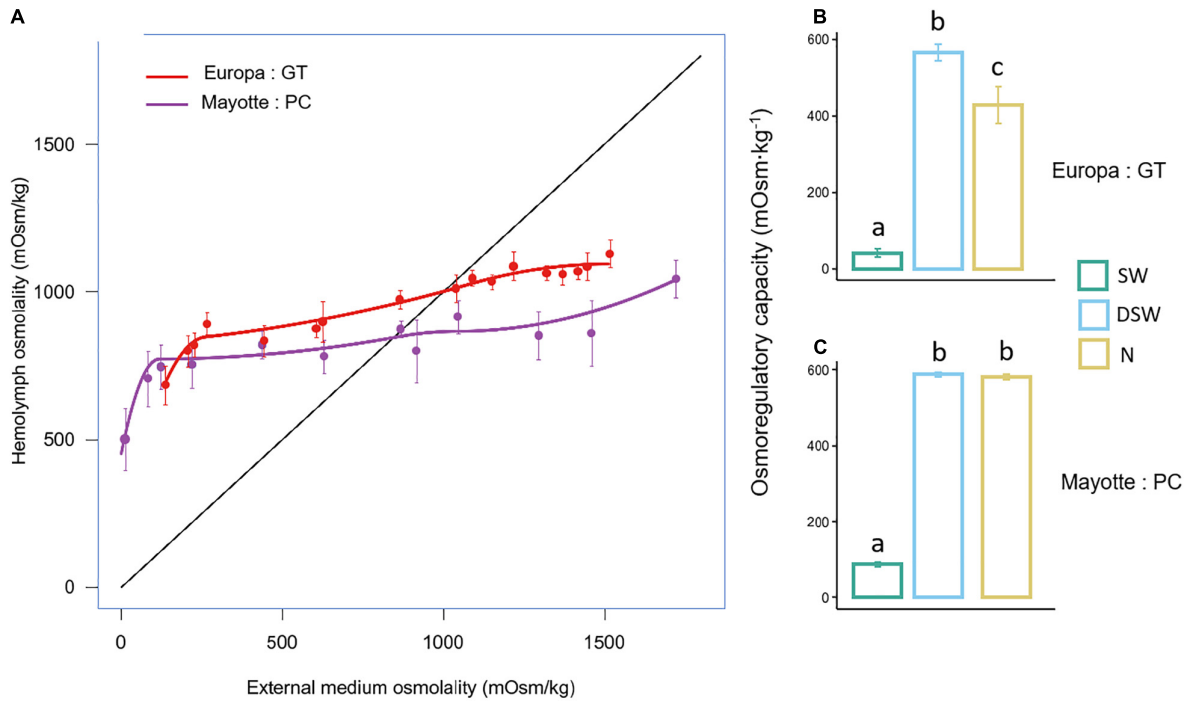

FIGURE 3 | (A) Variations in hemolymph osmolality in relation to the salinity of the external medium of GT and PC. **(B,C)** Osmolaregulatory capacity of GT and PC, respectively, after 96 h exposition to seawater (SW), diluted seawater (DSW) and 10 mg.L^{-1} ammonia-N (N). Values are means ± SEM (N = 7–8 per treatment). Different letters represent statistically significant differences between treatments at $p < 0.05$ obtained from a one-way ANOVA followed by Tukey *post hoc* comparisons.

during 96 h (**Figures 3B,C**). GT can maintain osmoregulatory capacity values of ∼50 mOsm.kg^{-1} in SW and ∼600 mOsm.kg^{-1} in DSW after 96 h, and these values are significantly different [**Figure 3B**, one-way ANOVA $F(2,20) = 89.44$, $p < 0.001$]. The same results are observed in PC with osmoregulatory values of ∼ 100 mOsm.kg^{-1} in SW and ∼ 600 mOsm.kg^{-1} in DSW [**Figure 3C**, one-way ANOVA, $F(2,20) = 1913$, $p < 0.001$]. However, a break in the curve is detected at low salinities, when it became increasingly difficult for the animals to osmoregulate. For GT, this break occurs at a higher salinity (∼260 mOsm.kg^{-1}) than for PC (∼118 mOsm.kg^{-1}). Moreover, PC was able to maintain the same osmoregulatory capacity in DSW with ammonia-N as in DSW (**Figure 3B**), whereas a significant decrease in osmoregulatory capacity is observed in GT exposed to ammonia-N compared with DSW (**Figure 3C**): crabs cannot maintain the haemolymph-seawater interval as in DSW.

Respiration Rate Across 96 h Diluted Seawater and Ammonia-N Exposures

Respiration rate measurements are depicted in **Figure 4A** for GT and **Figure 4B** for PC. They include temporal responses for each treatment at T-1h (SW), T0h, T24h, and T96h. In GT, a significant effect ($p < 0.001$) of exposure time on O_2 consumption (μmol O_2·m^{-1} g^{-1}) was observed. The interaction between treatment and exposure time was significant [$\chi^2(6, N = 31) = 16.831$, $p < 0.01$]. *Post hoc* multiple comparisons on the interaction terms showed that crabs exposed to ammonia-N significantly increased their respiration rate after 96 h of exposure compared to the other time steps and with DSW (**Figure 4A**). No effects of the osmotic or pollutant shock was observed immediately after exposure (T0h compared to T-1h).

In PC, no significant effect of treatment or exposure time was found, but their interaction was significant [$\chi^2(6, N = 32) = 44.5581$, $p < 0.01$]. *Post hoc* multiple comparisons on the interaction terms showed that crabs exposed to SW significantly decreased their respiration rate after 96 h exposure compared with the other time steps (**Figure 4B**). Crabs exposed to DSW progressively increased their respiration rate until 96 h (significant difference between T-1h and T96h and between T0h and T96h). At 96 h of exposure, a significant difference was observed between SW and DSW only.

Histological Analyses

The histology of the three main cell types of the pancreatic tubules of GT and PC is presented in **Figure 5I**: (1) R-cells (Restzellen, resorptive cells), the most abundant cell type in the hepatopancreas; (2) F-cells (Fibrillenzellen, fibrillar cells), which have a columnar shape and basophilic cytoplasm with an intense staining; (3) mature B-cells (Blasenzellen, blister-like cells), which are the largest cells showing large subapical vacuoles.

In GT, no effect of treatment was observed for all parameters measured in the hepatopancreas (One-way ANOVAS, **Table 1**, **Figures 5A–D**). In PC, a significant effect of treatment ($p < 0.01$) was reported regarding the percentage of B-cells vacuolization

in hepatopancreas tubules. Tukey *post hoc* showed an increased vacuolization process in DSW and ammonia-N compared to SW (**Figure 5H**).

No effect of treatment was observed in the gills of GT across the three levels of longitudinal axis (apical, central, and basal) (one-way ANOVAs, **Table 2** and **Figure 6A**). However, in PC, a significant effect of treatment ($p < 0.0001$) was reported at each level of the longitudinal axis (one-way ANOVAs, **Table 2**). Tukey *post hoc* tests revealed that the gill epithelium of crabs exposed to ammonium for 96 h was significantly thicker than those exposed to SW or DSW.

DISCUSSION

Osmoregulation and Ammonia-N Excretion

Osmoregulation is one of the key regulatory functions in aquatic organisms and represents an excellent indicator of the physiological status of crustaceans (Lignot et al., 2000). Crustaceans can be classified as strong, moderate, or weak osmoregulators depending on their capacity to tolerate low salinity and the magnitude of the hemolymph-seawater osmotic difference (Henry et al., 2012). Results indicate that both PC and GT are strong osmoregulators, but the breaking point in the osmolality curve (**Figure 2**) occurs at a higher salinity in GT compared to PC. Therefore, the physiological osmoregulatory limit toward low salinity for GT is reached before PC. Also, they do not share the same isosmotic point. It is higher in GT (∼1,000 mOsm.kg^{-1}) than in PC (∼830 mOsm.kg^{-1}). Similar results have been found in sympatric fiddler crabs living under different salinity conditions in the Gulf of Mexico (Thurman, 2004). Hypersaline species such as *Leptuca subcylindrica* and *Minuca rapax* are isosmotic for media between 840 and 940 mOsm.kg^{-1} (26–30‰) whereas, freshwater/brackish water species such as *Minuca longisignalis* are isosmotic at 732 mOsm.kg^{-1} (23‰). The ability to maintain hemolymph osmolality at different environmental salinities is well correlated with the habitat of the species (Lin et al., 2002), but may also depends on the species physiology itself. Consequently, a species living near estuaries or river mouths must be highly tolerant to salinity variation (hypothesis 1). Ecological knowledge about habitat preferences and distribution for both studied species tend to confirm this hypothesis. GT usually inhabits exposed, lower intertidal sandy or mud flat shores fringed with mangrove forest, coral rubble or rock (Dawn and Frith, 1977; Koga et al., 2000). This species is also considered as very marine, often living on shore and never near muddy river mouths (Crane, 1975). It has the largest longitudinal range of fiddler crabs, spanning the entire breadth of the Indian Ocean and Western Pacific subrealms, as described by Rosenberg (2020). PC does not live close to the open sea but near high-tide levels on the muddy banks and flats of mangrove estuaries, close to the mouth of streams or rivulets (Crane, 1975). It is one of the dominant species living in the study site of Mayotte and is one of the three endemic species from the East Africa Province (as defined by Rosenberg, 2020). This province also comprises three other species extended into other provinces, including GT. Thus, both species can be found in Europa and Mayotte islands even if they do not share the same habitat level and salinity conditions.

Since benthic crustaceans can frequently experience elevated levels of localized ammonia-N when they bury in sediments with high organic contents (Weihrauch et al., 1999), it is believed that this necessitates an adaptive mechanism to remove excessive ammonia-N build-up within their hemolymph (Weihrauch et al., 2004; Romano and Zeng, 2012). Ammonia excretion has been widely studied in aquatic crustaceans and it is established that this function is intimately linked to the gills and their osmoregulatory function (see Weihrauch et al., 2004 for a complete description of the model).

The mechanisms for ammonia-N excretion in bimodal crabs are very diverse and complex (for a review, see Weihrauch and O'Donnell, 2017), as many membrane transporters are involved mainly in the anterior and posterior gills as well as in the antennal gland. The sodium pump Na^+/K^+-ATPase (NKA), K^+-channels and Na^+/H^+ exchangers (NHE) are among the key membrane proteins involved although more transporters could be at play (Masui et al., 2002, 2009; Lucu and Towle, 2003; Gonçalves et al., 2006; Freire et al., 2008; Weihrauch and O'Donnell, 2017). As already described, a number of these transporters playing a crucial role in *trans*-epithelial ammonia excretion are also involved in the ionoregulatory machinery (Weihrauch and O'Donnell, 2015, 2017). The best example for this is NKA, one of the key transporters responsible for energizing active NaCl transport processes in invertebrates and vertebrates alike (Larsen et al., 2014). The NH_4^+ pump (often referred to NH_4^+/K^+-ATPase) is equally important. However, this process reaches its limits when crustaceans are exposed to excessively high amounts of ammonia-N, generally resulting in reduced hemolymph Na^+ levels (Romano and Zeng, 2012). This phenomenon has been observed in several species living under various salinity conditions: the American clawed lobster, *Homarus americanus* (Young-Lai et al., 1991), the Kuruma shrimp, *Marsupenaeus japonicus* (Chen and Chen, 1996), the burrowing crab *Neohelice granulata* (Rebelo et al., 1999), the freshwater crayfish *Pacifastacus leniusculus* (Harris et al., 2001) and the mud crab *Scylla serrata* (Romano and Zeng, 2007a), amongst others. We can relate these data to our results on osmoregulatory capacity: increased ammonia-N excretion could induce a leak in Na^+ ions followed by a decrease in OC for GT, but not for PC. This may suggest that PC could set-up physiological mechanisms that allow a tolerance capacity to high ammonia-N levels in DSW. However, GT could not (rapidly) mobilize such tolerance. In most Crustaceans (i.e., *H. americanus* and *P. japonicus*), a decrease in hemolymph Na^+ levels is directly linked with reduced hemolymph osmolality and/or OC, but this is not observed in the euryhaline mud crab *S. serrata* (Romano and Zeng, 2007a). These authors propose that this crab species relies on other mechanisms to maintain osmotic pressure even in case of Na^+ reduced levels, as for example, by increasing the organic osmolytes such as hemolymph free amino acids (FAA). One can hypothesis that the difference observed in OC between GT and PC may result in different abilities to excrete ammonia-N (through NKA, NH_4^+/Na^+ exchanger, and/or other

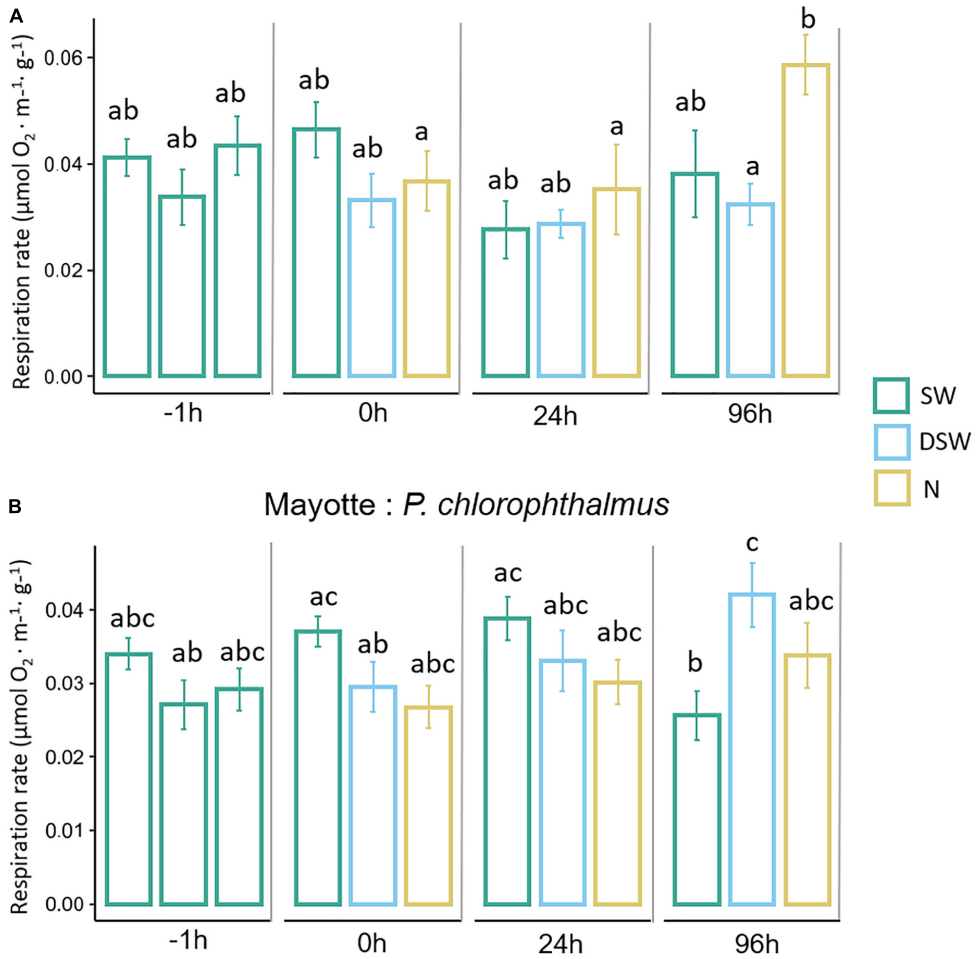

FIGURE 4 | Resting metabolic rate of GT **(A)** and PC **(B)** in SW, DSW and N at T-1h, T1h, T24h, and T96h exposure. Values are mean ± SE. Different letters indicate significant two-way interaction effects of treatment and time after Wald Chi-square test and pairwise multiple comparisons (Bonferroni corrected).

transporters). With ammonia-N in DSW, GT may exhibit lower NKA activity, as reported in the blue swimmer crab *Portunus pelagicus* exposed to ammonia-N at low Na^+/K^+ ratio (Romano and Zeng, 2011), leading to lower Na^+ levels in the hemolymph compared to PC. A possible explanation could be that PC may better cope with high levels of ammonia-N in its hemolymph than GT, by maintaining the activity level of its membrane transporters. Measuring ammonia-N, ion concentration and pH levels in the hemolymph would help to confirm this hypothesis.

Implications of Osmoregulation Strategies in the Whole Metabolism of Both Species

Increased energy expenditure for osmoregulation and ammonia-N excretion may be reflected by increased metabolic indicators such as oxygen consumption (Gomez-Jimenez et al., 2004).

Our results showed no effects of DSW in respiration rate of GT across the 96 h of exposition compared to the controls in DSW. In PC, respiration rate progressively increases in DSW, whereas O_2 consumption in SW progressively decreased until 96 h of exposure. Euryhaline crabs are able to compensate for diffusion loss of ions in dilute media by active uptake of gill epithelium (Genovese et al., 2004). The most energy-demanding ion transport mechanism is the active transport driven by the NKA (Lucu and Towle, 2003), with active ion pumping in specialized epithelial cells that are also mitochondria rich (Freire et al., 2008; McNamara and Faria, 2012). Ammonia-N excretion and hyper-osmoregulation mainly rely on these mechanisms (Weihrauch et al., 2004) which generally increases when estuarine/marine crustaceans are exposed to diluted seawater (reviewed in Romano and Zeng, 2012). Based on the large loss of salt in diluted media, these crustaceans must invest considerable amounts of energy in active transport/uptake of salt in order to compensate for the large rates of diffusive salt loss (Henry et al., 2012). When osmotic stress is coupled with ammonia-N stress, an increase in O_2 consumption is observed in GT after 96 h exposure, but this was not observed in PC.

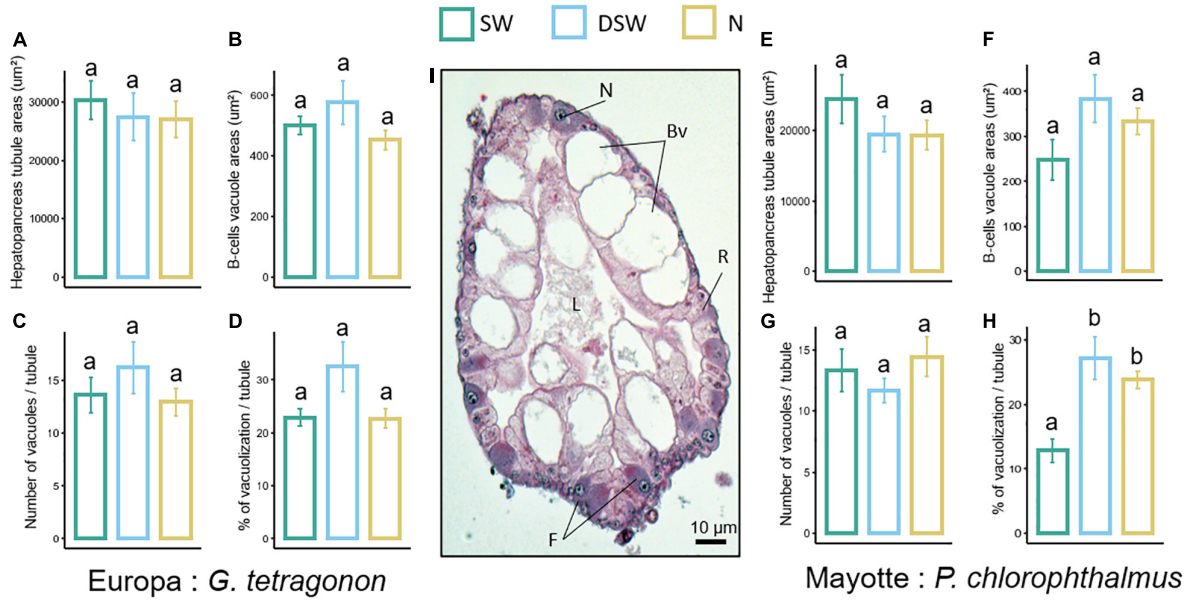

FIGURE 5 | Histological analyses from transverse sections of different tubules of GT **(A–D)** and PC **(E–H)** hepatopancreas exposed to seawater (SW), diluted seawater (DSW) and 10 mg.L^{-1} ammonia-N (N). Measurements and counts were carried out on randomly distributed tubules per individual. Data were averaged per individual. **(A,E)** Areas of the tubules expressed as mean per individual; **(B,F)** B-cell vacuole areas expressed as mean per tubule per individual; **(C,G)** number of B-cell vacuoles in the tubule; **(D,H)** percentage of B-cell vacuolization area per tubule; **(I)** light microscopy illustration of a tubule cross section (GT). The photograph shows a tubule epithelium containing absorbing R-cells (R), enzyme synthetizing F-cells (F), and tubule-clearing B-cells (Bv). L, lumen; N, nucleus. Values are means ± SEM (N = 3–8 per treatment). Different letters represent statistically significant differences between treatments at $p < 0.05$ after Wald Chi-square test and multiple comparisons (Tukey).

This could be directly linked to the OC decrease observed in this species. An increased energy demand is likely to occur in case of ammonia-N prolonged exposure. For instance, GT could rely on different ionic transport mechanisms without necessarily modifying its gill epithelial thickness (as discussed in Section "Histological Changes to Explain *Paraleptuca chlorophthalmus* and *Gelasimus tetragonon* Differential Ammonia-N Tolerance"). Even with an increased uptake of O_2, it is possible that the maintenance of GT hydromineral balance and ammonia-N excretion function is less efficient when facing these stressors than in PC. This could highlight a physiological trade-off because of the lack of other adaptive mechanisms, but also due to any physiological differences between the two species. In PC maintained in DSW, the increase in O_2 uptake could be due to an increased energy demand for osmoregulation (among others). However, a lower oxygen consumption in DSW with ammonia-N could suggest an increased anaerobic metabolism or a different compromise between gas exchange and osmoregulation at the gill surface (aka the osmo-respiratory compromise) (Robertson et al., 2015). A clear functional specialization has been reported for several crab species, posterior gills being the main effectors of the "compensatory" process, while anterior gills are mostly involved in gas exchange (Taylor and Taylor, 1992; Pequeux, 1995; Genovese et al., 2004). Anterior gills are also known to be actively involved in ammonia excretion (Weihrauch et al., 1998, 1999) and in some hyper-regulating species such as green shore crab, active ammonia excretion rates being lower in posterior osmoregulatory gills compared to anterior gills (Weihrauch et al., 1998). These results indicate that osmoregulatory processes are not directly linked to ammonia excretion and that excretion of toxic ammonia could proceed independently of other physiological processes (Weihrauch and O'Donnell, 2017). Large areas in the lamellae of the posterior gill epithelium reveals thick ion-transporting cells with well-developed basolateral membranes associated with numerous mitochondria in several species of euryhaline crabs such as *Neohelice granulata* (previously *Chasmagnathus granulatus*) (Genovese et al., 2004). Most of the NKA activity is generally restricted to specific areas of posterior gill lamellae lined by this kind of epithelium (Towle and Kays, 1986).

Histological Changes to Explain *Paraleptuca chlorophthalmus* and *Gelasimus tetragonon* Differential Ammonia-N Tolerance

In DSW, no changes were observed in the thickness of the gill epithelium for the two species (**Figures 6A,B**). However, when exposed to ammonia-N for 96 h, branchial epithelium thickness of PC significantly increases along the longitudinal axis, whereas this does not occur for GT. An increase in osmoregulatory epithelial patches surface of posterior gills in crabs exposed to either dilute or hyper-saline water (i.e., when crabs osmoregulate) has already been observed (Genovese et al., 2000; Lovett et al., 2006). The authors suggest that these gills could be involved both in active ion uptake and active excretion of ions depending on

TABLE 1 | Results of analysis of deviance for histological measurements from transverse sections of different hepatopancreas tubules of *G. tetragonon* and *P. chlorophthalmus* as a function of treatment.

A	Hepatopancreas tubule areas (μm^2)					
Factor	**DF**	χ^2		***P***		
	GT-PC	**GT**	**PC**	**GT**	**PC**	
Treatment	2	0.9819	1.4586	0.6121	0.4822	
B	Number of vacuoles/tubules					
Factor	**DF**	χ^2		***P***		
	GT-PC	**GT**	**PC**	**GT**	**PC**	
Treatment	2	1.4951	1.3823	0.4735	0.501	
C	B-cells vacuole areas (μm^2)					
Factor	**DF**	χ^2		***P***		
	GT-PC	**GT**	**PC**	**GT**	**PC**	
Treatment	2	1.0779	4.4643	0.5834	0.1073	
D	% of vacuolization/tubule					
Factor	**DF**	χ^2		***P***		
	GT-PC	**GT**	**PC**	**GT**	**PC**	
Treatment	2	3.1161	9.5381	0.2105	**0.008489**	**

*GT, Gelasimus tetragonon (Europa Island); PC, Paraleptuca chlorophthalmus (Mayotte). Wald Chi-square tests are performed for testing the significant differences of fixed categorical variables in GLMM (N = 7–8 per treatment for 3 treatment group). Statistically significant effects are indicated by asterisks: **p < 0.01 and appear in bold.*

TABLE 2 | Results of analysis of variance for gill epithelial thickness measured on different parts of the longitudinal axis of the gill one-way ANOVAs).

A	Gill epithelial thickness – apical part								
Factor	**DF**		**MS**		***F***		***P***		
	GT	**PC**	**GT**	**PC**	**GT**	**PC**	**GT**	**PC**	
Treatment	2	2	0.8836	20.635	0.551	15.36	0.579	**3.68E-06**	***
Residuals	57	63	1.6029	1.343					
B	Gill epithelial thickness – central part								
Factor	**DF**		**MS**		***F***		***P***		
	GT	**PC**	**GT**	**PC**	**GT**	**PC**	**GT**	**PC**	
Treatment	2	2	2.054	14.839	1.503	13.79	0.231	**1.07E-05**	***
Residuals	57	63	1.367	1.076					
C	Gill epithelial thickness – basal part								
Factor	**DF**		**MS**		***F***		***P***		
	GT	**PC**	**GT**	**PC**	**GT**	**PC**	**GT**	**PC**	
Treatment	2	2	2.057	20.844	0.841	11.37	0.437	**6.08E-05**	***
Residuals	57	63	2.447	1.833					

GT, Gelasimus tetragonon (Europa Island); PC, Paraleptuca chlorophthalmus (Mayotte).
*N = 7–8 per treatment for 3 treatment group. Statistically significant effects are indicated by asterisks: ***p < 0.001 and appear in bold.*

the medium salinity, especially with the progressive increase of NKA activity during 6 days observed in crabs exposed to dilute seawater (Lovett et al., 2006). In PC, this response may be directly related to a strategy allowing increased ammonia-N excretion and osmoregulation may be through NKA increased activity, as discussed above. By increasing the thickness of the basolateral epithelium of posterior gill cells, PC may increase the number of ion transporters (mainly NKA) and mitochondria allowing, therefore, an increase in its osmoregulatory and ammonia-N excretion capacities. The increase in epithelial thickness does not occur in DSW although a higher metabolic rate is observed in this condition. Therefore, PC could rely on two different strategies depending on the nature and level of stress (increased metabolic rate in DSW and/or enhanced ion transport mechanisms when exposed to ammonium by the lengthening of the basolateral epithelium), whereas GT cannot exhibit such plasticity regarding ammonia-N exposure and increases its metabolic rate.

However, though not quantified, no major disruption of cellular structure has been observed in either species exposed to DSW or Ammonia-N. In Romano and Zeng (2007b), Ammonia-N exposure for 96 h lead to different dose-dependent disruptions of gills in *P. pelagicus*. At 10 $mg.l^{-1}$ ammonia-N (a similar concentration as in the present study), gill lamellae showed local infiltrations of hemocytes with normal lamellae structures whereas 60 $mg.l^{-1}$ ammonia-N generated extensive hemocyte infiltrations, disrupted pillar cells and lamellae collapses.

In Crustaceans, the hepatopancreas is a key metabolic organ, expressing a variety of transporters (Ahearn et al., 1992). Its numerous tubules are involved in food digestion, detoxification of xenobiotics and nutrient absorption (Ceccaldi, 1998; Zilli et al., 2003; Ortega et al., 2014). Histological analyses of these tubules revealed few changes for the B-cells in both GT and PC (**Figures 5A–G**). Even if their role is still discussed, B-cells can be considered to have two functions: elimination of waste material and intracellular digestion of nutrients (Vogt, 1994). The supranuclear vacuoles resulting from the coalescence of subapical vacuoles take the form of huge lysosomes (Vogt, 1993) and their content can be discharged into the lumen by holocrine secretions (Nott et al., 1985). Our results showed a higher vacuolization in PC exposed to Ammonia-N that could be linked to an increased intracellular digestion process through B-cells vacuoles, as it has been found in a Sesarmid crab species exposed to domestic wastewater (Mégevand et al., 2021). This increase in activity would potentially lead to an increased energy expenditure but in this case, our hypothesis is that ammonia-N could have a limited effect on the hepatopancreas functioning. As mentioned by Chu (1987), the hepatopancreas overall contribution to osmoregulatory ion uptake is believed to be minimal, whereas gills are the main site for respiration, osmoregulation, ammonia-N excretion, these gills being the main organs involved for the overall organismal adaptation to changing conditions. In Ocypodids such as PC and GT,

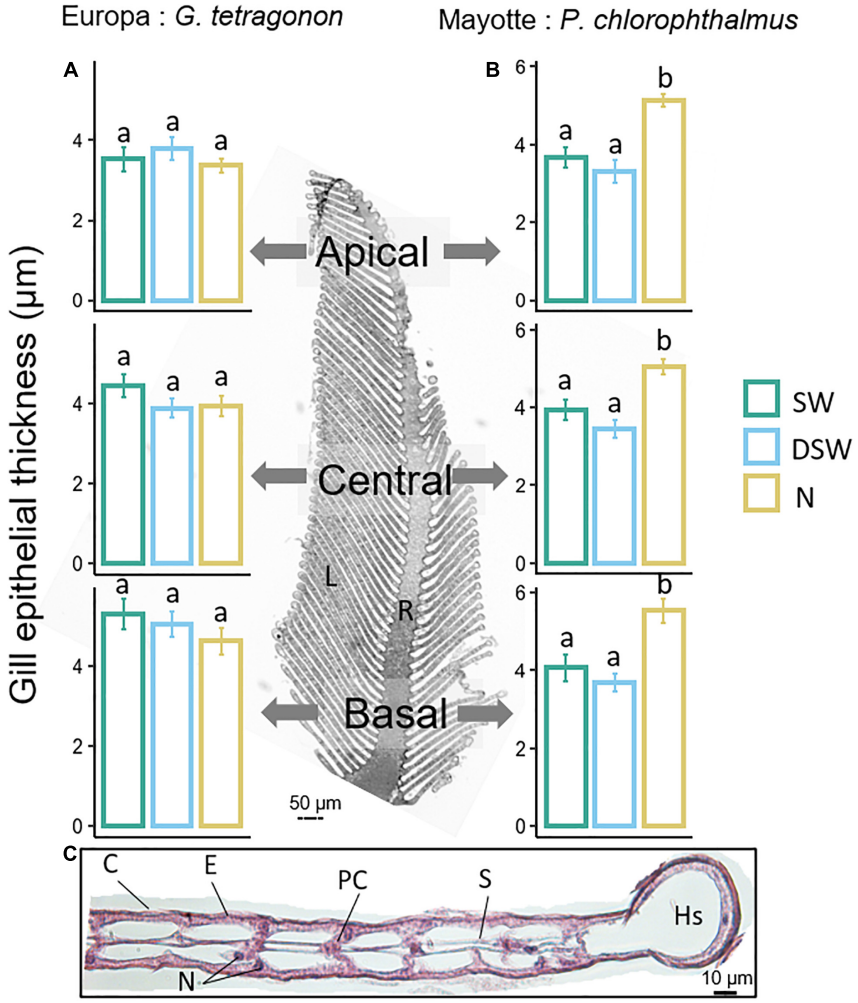

FIGURE 6 | Histological analyses from transverse sections of GT and PC posterior gills exposed to seawater (SW), diluted seawater (DSW) and 10 mg.L^{-1} ammonia-N (N). The photograph illustrates a histological cross section of a posterior gill through the raphe (central part, R) and the lamellae (L). **(A,B)** Epithelial thickness measurements of gill lamellae across the longitudinal axis of posterior gills of GT and PC. Values are means ± SEM (N = 3–8 per treatment). Different letters represent statistically significant differences between treatments at $p < 0.05$ obtained from a one-way ANOVA followed by Tukey *post hoc* comparisons. **(C)** Histological section of a gill lamella of *Gelasimus tetragonon* (cross section). C, cuticle; E, epithelium; N, nucleus; PC, pillar cell; S, septum; Hs, hemocoelic space.

the study of the antennal gland would also be of interest in order to understand ammonia excretion mechanisms. As shown for several fiddler crabs such as *Minuca pugnax* or *Leptuca pugilator*, ammonia is known to be actively transported across the duct and excreted in the primary urine which contains a high concentration of ammonium (Green et al., 1959; De Vries et al., 1994). Other ecomorphological markers in addition to those used on the hepatopancreas and gills would be interesting to study as indicators of adaptation to changing environments. For example, Lim and Goh (2021) demonstrated that the setae present on the maxillipeds are great indicators of adaptation, especially for efficient food extraction regarding sediment quality. Fiddler crabs with greater diversity and density in setae could be able to adapt to more variable habitats since their ability to extract food from different sediment types would be better.

Coping with an aquatic environment with low salinity and elevated ammonia-N is highly species- and habitat-specific. Therefore, a fully euryhaline species such as PC, that is adapted to nutrient-rich environments (Gross, 1982; Henry et al., 2012) is likely to be less vulnerable to nutrient enrichment and may be able to develop other coping mechanisms. However, this study focuses on potential short-term acclimation capacities. It does not take into account any possible environmental determinism that could arise from micro-evolution after prolonged exposure to ammonia-N enriched conditions (in Mayotte) or differential abiotic factors such as eutrophic or oligotrophic environments.

A research program with multi-generational expositions of the two populations would be of high interest, especially for understanding ecosystem health and ecological risk implications on a longer timescale (although technically complicated to implement).

Ecosystem and Risk Assessment Implications

Acclimatory and adaptive capacities to different environments induce species-specific responses among Decapod crustaceans that rely on various mechanisms to maintain their metabolism (Weihrauch et al., 1999; Henry et al., 2012; Romano and Zeng, 2013).

However, brackish and intertidal environments are among the most stressful aquatic biotopes and the establishment of Crustacean communities in such habitats suppose highly adapted physiological features (Gilles and Pequeux, 1983; Charmantier et al., 2002) to cope with low and varying salinities (Henry et al., 2012), as well as constantly changing environments. For example, it has been shown by Yong and Lim (2021) that ocypodid crabs such as *Ocypode gaudichaudii* possess a feeding plasticity when confronted to different habitat types, which is related to their burrowing and foraging strategies. In the case of our study, salinity variations and nitrogen enrichment phenomena could also induce different levels of adaptation in feeding and foraging habits, which would be interesting to study in both species in parallel with the individual physiology.

In the present study, PC appears to have a better physiological tolerance to decreased salinity compared to GT, even if GT is a relatively strong osmoregulator (hypothesis 1). Ammonia-N triggers short-term acclimation in PC that was not observed in GT suggesting that this species could be more vulnerable to anthropogenic discharges (hypothesis 2). GT copes with 96 h ammonia-N exposure through increased metabolic rate to excrete this product, while struggling to maintain its osmoregulatory balance. However, this response does not occur in DSW condition without ammonia-N. This suggests that some cumulative deleterious effects under Ammonia-N exposure and osmotic stress occur for this species (hypothesis 3).

Ammonia-N was used in this study as a first indicator of anthropogenic effluents, but it is more likely that crab communities are confronted with different contaminants with combined effects (e.g., complex and hypoxic wastewater), often consisting of repeated exposures. Worldwide, effects of domestic effluent discharges (controlled or not) on crab community assemblage have been observed in several different mangroves with various results (Wear and Tanner, 2007; Penha-Lopes et al., 2009; Wickramasinghe et al., 2009; Capdeville, 2018). The results of the present study point (at least) to a short-term physiological acclimation to salinity variation and pollution for species such as PC, acclimated to euryhaline and anthropized conditions (already proposed in Mégevand et al., 2021, submitted), but it has been demonstrated that individual abundance for this species decreased in chronically WW impacted areas (Capdeville et al., 2018). If GT is unable to develop short-term response to salinity and ammonia-N inputs, it is likely that this species would be even more sensitive to longer exposure to pollution, or to more complex contaminants such as hypoxic WW. If the population decline of engineering species can be dreadful for the functioning of freshwater-influenced mangroves (e.g., Mayotte), what about isolated and pristine ecosystems like Europa Island? What would be the acclimation potential of representative species like GT in the event of anthropogenic effluents? In view of the results obtained in this study and those obtained during the various works mentioned above, it is possible to be pessimistic about the ecosystem risks incurred by a pristine mangrove such as that of Europa if it were to face waters discharges of anthropogenic origin.

CONCLUSION

The two crab species studied here showed different physiological plasticity that led to different tolerance to salinity variations and ammonia-N exposure. In accordance with our first hypothesis, *P. chlorophthalmus* (Mayotte Island) has a better osmoregulatory capacity in diluted seawater than *G. tetragonon* (Europa Island), which remains, however, a strong hyper-hypo-osmoregulator. Exposure for 96 h to ammonia-N at a concentration of 10 $mg.l^{-1}$ in diluted seawater (5ppt) generates branchial morphological acclimation in PC most likely in order to increase the osmoregulatory and Ammonia-N excretion functions. These changes do not occur in GT, this species experiencing an increase in oxygen consumption over time as its osmoregulatory capacity decreases (hypothesis 2). The two species do not have the same tolerance to short-term nitrogen inputs (as a simulation of anthropogenic presence) (hypothesis 3). Burrowing crabs ensure crucial ecological functions associated with their bioturbation activity. A change in crab community composition will not ensure the maintenance of these functions as functional redundancy may not happen. Consequences in terms of mangrove functioning and vulnerability are difficult to predict and linked to many others parameters. However, our study emphasizes the importance of considering small/medium scale ecophysiological indicators of species vulnerability, especially when developed in engineer species, to answer large-scale questions of ecosystem risk assessment.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LM: conceptualization, methodology, investigation, formal analysis, and writing – original draft. DT: conceptualization, methodology, investigation (osmoregulation curves and osmoregulatory capacity), formal analysis, and writing – review and editing. CL'É: investigation (histological analyzes). SH: methodology (histological analyzes), investigation (histological analyzes), and writing – review and editing. EC: methodology, investigation (molecular analysis of rRNA sequences), and writing – review and editing. TL'H: investigation (histological experiments) and writing – review and editing. J-HL: supervision, conceptualization, investigation, and writing – review and editing. ES: project administration, funding acquisition,

supervision, conceptualization, investigation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.839160/full#supplementary-material

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Does IGF-1 Shape Life-History Trade-Offs? Opposite Associations of IGF-1 With Telomere Length and Body Size in a Free-Living Bird

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Hormonal pathways have been proposed to be key at modulating how fast individuals grow and reproduce and how long they live (i.e., life history trajectory). Research in model species living under controlled environment is suggesting that insulin-like growth factor 1 (IGF-1), which is an evolutionarily conserved polypeptide hormone, has an important role in modulating animal life histories. Much remains, however, to be done to test the role played by IGF-1 in shaping the phenotype and life history of animals in the wild. Using a wild long-lived bird, the Alpine swift (*Tachymarptis melba*), we show that adults with higher levels of IGF-1 had longer wings and shorter telomeres. Hence, telomeres being a proxy of lifespan in this species, our results support a potential role of IGF-1 at shaping the life-history of wild birds and suggest that IGF-1 may influence the growth-lifespan trade-off.

Keywords: aging, IGF-1, life-history, lifespan, pace-of-life syndrome, telomeres, trade-off

INTRODUCTION

How fast an individual grows and reproduces and how long it lives are referred to as its life history trajectory (Flatt and Heyland, 2011). Although life history traits exhibit a wide range of variation among species, consistent patterns of covariation between traits are widespread (Gaillard et al., 1989). Such patterns of covariation are thought to be regulated by competitive allocation of limited resources (Stearns, 1992), although other proximate mechanisms have also been suggested (Harshman and Zera, 2007). Accordingly, hormonal pathways have been proposed to play a key role in regulating life-history trade-offs (Ricklefs and Wikelski, 2002). One candidate hormone is insulin-like growth factor 1 (IGF-1), which is an evolutionarily conserved polypeptide known to have effects on three key life-history traits: growth, reproduction, and survival (Swanson and Dantzer, 2014; Lodjak and Verhulst, 2020). Moreover, IGF-1 is suspected to be involved in the

regulation of the trade-offs among these three life-history traits, but this hypothesis has only been scarcely tested in free-living animals (Swanson and Dantzer, 2014; Lodjak and Verhulst, 2020).

In laboratory models (i.e., worms, flies, and mice), IGF-1 signaling and circulating levels are linked to the growth of somatic (Musarò et al., 2001; Yakar et al., 2002) and reproductive structures (Kaaks et al., 2000), gonadal function regulation (Bartke et al., 2013), and reproductive performance (Li et al., 2011). Moreover, the inhibition of IGF-1 signaling is associated with increased lifespan (Holzenberger et al., 2003), but the proximate mechanisms underlying this association are less well understood. Although IGF-1 levels are highly repeatable within individuals, 0.41–0.85 (Roberts et al., 1990; Yuan et al., 2009), they respond to environmental fluctuations on nutritional resource availability and temperature (Gabillard et al., 2003; Sparkman et al., 2009; Regan et al., 2020). Therefore, studies on the role played by IGF-1 on growth, reproduction, and survival, in natural settings may be particularly relevant. In free-living animals, ecological conditions have been shown to influence the direction of the links between IGF-1 and life-history traits. For example, in free-ranging garter snakes (*Thamnophis elegans*) the presence of a positive association between IGF-1 levels and adult body size is dependent on the habitat type (Sparkman et al., 2009). So far, general conclusions reached in laboratory models, on the positive association between IGF-1 with growth have been (at least partially) confirmed in several non-model species (inter-specific comparison, Lodjak et al., 2018). Moreover, although the number of studies evaluating the link between IGF-1 and lifespan in non-model species has been very limited, they mostly confirmed the negative association between IGF-1 and lifespan reported in model organisms, both at the interspecific (Swanson and Dantzer, 2014; Lodjak et al., 2018) and at the intraspecific level (Lewin et al., 2017, but see Chaulet et al., 2012; Lendvai et al., 2020). Still, the nature of the proximate mechanisms implicated remains elusive, and one of the putative candidates is oxidative stress (Holzenberger et al., 2003), which is known to accelerate the shortening of telomeres (Reichert and Stier, 2017), one of the hallmarks of aging (López-Otín et al., 2013).

Although understanding variation in lifespan is a major focus of evolutionary ecology, accurately measuring lifespan in the wild can be logistically challenging (Nussey et al., 2008). Remarkably, telomere length has been shown to be linked to life expectancy in non-model species (Wilbourn et al., 2018). Telomeres are non-coding sequences of repetitive DNA located at the end of linear chromosomes that shorten with each cellular replication (Backburn and Epel, 2012), in response to cellular stressors including oxidative stress (Reichert and Stier, 2017), and under increased metabolic demand (Casagrande and Hau, 2019). Both, IGF-1 and telomere length, have been shown to diminish with age (Moverare-Skrtic et al., 2009) and are linked to longevity (Deelen et al., 2013). The association between IGF-1 and telomeres has mainly been explored in laboratory animals and human patients exhibiting pathological disorder of their somatotropic axis as well as cancer cell lines (Aulinas et al., 2013; Matsumoto et al., 2015). While cross-sectional and correlative studies in humans and laboratory animals showed that IGF-1 and telomere length are positively related (Barbieri et al., 2009; Moverare-Skrtic et al., 2009; Yeap et al., 2019), studies conducted in cell lines found a negative association between these variables (Matsumoto et al., 2015; Matsumoto and Takahashi, 2016). Hence, the direction and strength of association between IGF-1 and telomeres is still equivocal, and much remains to be done to understand whether telomere dynamics is part of the mechanisms underlying the covariation observed between IGF-1 and lifespan.

The Alpine swift (*Tachymarptis melba*) is an insectivorous migratory bird that live up to 26 years of age and has a slow pace of life (i.e., late age at sexual maturity, only one reproductive attempt *per* year, and small clutch size *per* breeding attempt) (Bize et al., 2009). The fact that adult Alpine swifts with longer telomeres have higher survival rate (Bize et al., 2009) makes them a good model for exploring the links between IGF-1 and telomere length. We propose that, if IGF-1 is a proximate mechanism underlying trade-offs between growth and lifespan, then IGF-1 should show a positive association with body size and a negative relationship with telomere length. As effects of IGF-1 on size can be tissue specific (Sharma et al., 2012), and thus may affect the growth of different body parts in dissimilar ways, we measured wing length, sternum length and body mass as proxies of body size.

METHODS

Fieldwork was carried out in 2018 in two Alpine swift colonies located in Bienne and Solothurn, Switzerland, where nestlings have been ringed each year since at least 1968 and adults have been subjected to an individual based study since 2000 (Bize et al., 2009). Forty-three adult Alpine swifts were captured on their nests or roosting during the breeding season. After capture, each adult was weighed with a digital scale to the nearest 0.1 g, the same person (PB) measured their wing with a ruler to the nearest mm and their sternum with a caliper to the nearest 0.1 mm. Wing length was measured as the distance on the closed wing from the foremost extremity of the carpus to the tip of the longest primary feathers; the wing being flattened and straightened to give maximal wing length. Sternum was measured from one end to the other of the keel. We collected a 200 μL blood sample from the foot vein using heparinized microvette tubes (Sarstedt, Germany) to quantify telomere length and IGF-1 levels. Blood samples were kept on ice in the field before being centrifuged at 10 000 $g \times 10$ min to separate plasma from red blood cells, and then stored at −80°C until laboratory analyses. The Alpine swift is a monomorphic bird species, and thus adults were genetically sexed (Bize et al., 2005). The exact chronological age was known for 38 birds that had been ringed as nestlings (range: 2–17 years).

Telomere Length

Multiplex quantitative PCR was used to quantify relative telomere length (RTL). Full protocols for genomic DNA extraction using nucleated red blood cells and of multiplex qPCR description adapted for swifts are available in Criscuolo et al. (2017). The concentration and purity (presence of residual proteins or

solvents) of the extracted DNA were checked with a Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States). The quality and the integrity of DNA were confirmed by gel electrophoresis on 1% agarose gels stained with ethidium bromide (checking for the absence of DNA smears corresponding to degraded DNA). Our 43 adults' RTL were measured within a batch of chick and adult samples (total 109 samples) measured in duplicates over one 384 wells plate. Amplification efficiencies of control and telomere sequences were of 99.6% (identical for both sequences) and r^2 of dilution curves of 0.97 and 0.98, respectively. Based on duplicates within plates, intra-class correlation coefficient for final RTL values was 0.689.

Insulin-Like Growth Factor-1

IGF-1 concentrations were measured from 10 μl of plasma using an enzyme-linked immunosorbent assay (for further details see Mahr et al., 2020). We used chicken plasma in quadruplicates to determine intra- (5.3%) and inter-assay coefficient of variation (10.4%). We validated the assay for the Alpine swifts by showing that serially diluted plasma samples were parallel with the standard curve.

Statistical Analyses

Statistical analyses were performed in R version 4.0.0, using the R package lmerTest (Kuznetsova et al., 2017; R Core Team, 2021). A preliminary analysis exploring the effect of sampling date, hour, and breeding site on IGF-1 levels showed that only sampling date accounted for variation in IGF-1 levels; sampling date was therefore retained as a covariate when analyzing variation in IGF-1. Firstly, we tested for an association between IGF-1 and wing length, sternum length and/or body mass by fitting a general linear model with IGF-1 as response variable and all three biometric traits, plus sampling date, as explanatory variables (model 1). Variance inflation factors in this first model were below 1.24 indicating no collinearity issues between biometric traits. Secondly, we investigated how IGF-1 (response variable) was related to telomere length by including as explanatory variables telomere length while controlling for possible confounding effect of chronological age and traits identified as significant in our model 1. Sample sizes vary across analyses due to missing values in some of the traits.

RESULTS

Plasma levels of IGF-1 in adult Alpine swifts were positively associated with wing length (**Figure 1A** and **Table 1A**) but not related to body mass and sternum, those results being obtained after controlling for the date of sampling (**Table 1A**). Adults sampled later in the season had lower IGF-1 levels (**Table 1**). Hence, wing length and sampling date were included as covariates in the follow-up analysis. Adult birds with higher IGF-1 levels had shorter telomeres (**Figure 1B** and **Table 1B**), after controlling for the date of sampling, wing length, and chronological age (**Table 1B**).

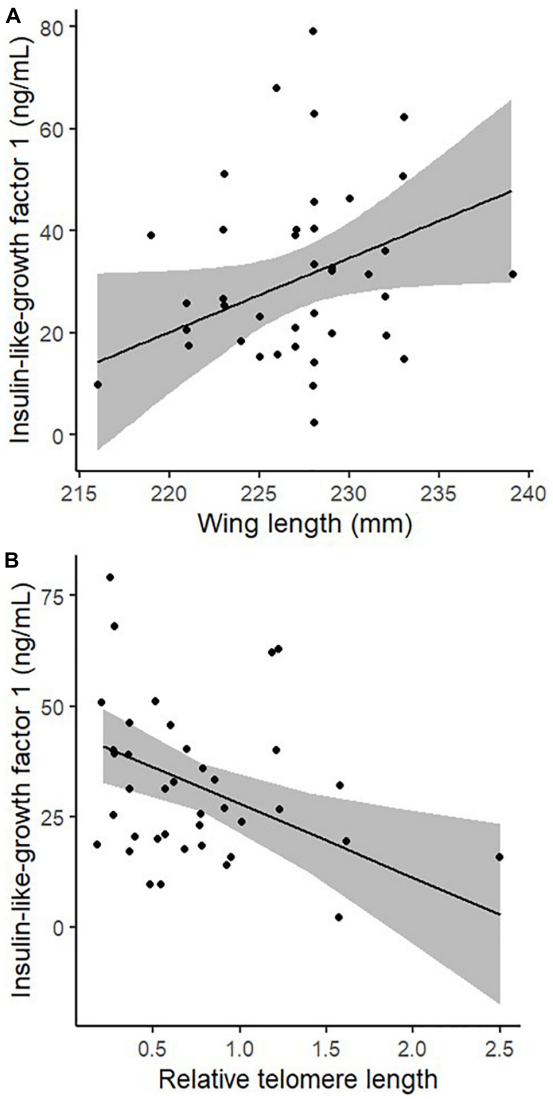

FIGURE 1 | Panel **(A)** shows the relationship between wing length and plasma levels of insulin-like growth factor 1, in adult Alpine swifts. Panel **(B)** presents the association of relative telomere length and plasma levels of insulin-like growth factor 1, in adult Alpine swifts.

DISCUSSION

Overall, as predicted, we found that circulating levels of IGF-1 were positively associated with wing length and negatively to telomere length, in adult Alpine swifts. Those opposite associations of IGF-1 with body size and telomere length support the idea that IGF-1 may regulate, at least partially, the life-history of this long-lived bird species.

Our results show that adult birds with higher levels of IGF-1 had longer wings, which matches recent findings in adult bearded reedlings (*Panurus biarmicus*) showing a positive association of between IGF-1 levels and tail feather length (Mahr et al., 2020) and in nestling pied flycatchers (*Ficedula hypoleuca*) showing a positive association between IFG-1 and growth and size at

TABLE 1 | Linear models showing positive association between plasma levels of insulin-like growth factor 1 and wing length (model 1), and negative relation between plasma levels of insulin-like growth factor 1 and relative telomere length (model 2), in adult Alpine swifts.

(A) Model 1: Linking IGF-1 to body size			
Variable	***Estimate ± SE***	***F***	***P***
Wing	1.46 ± 0.71	4.21	0.049
Sternum	2.34 ± 2.45	0.92	0.34
Body mass	−1.06 ± 0.59	3.20	0.08
Sampling date	−1.14 ± 0.51	4.91	0.03
Residual DF		30	
(B) Model 2: Linking IGF 1 to telomere length			
Variable	***Estimate ± SE***	***F***	***P***
Telomere length	−16.62 ± 5.56	8.92	0.006
Wing	0.83 ± 0.63	1.75	0.20
Chronological age	−0.54 ± 0.70	0.59	0.45
Sampling date	−0.68 ± 0.49	1.93	0.18
Residual DF		29	

Table shows results from the initial model, no stepwise deletion was performed.

fledging (Lodjak et al., 2016). The positive association between IGF-1 and wing length in adult swifts may be caused either by the effects of IGF-1 on wing bone growth early in life and/or primary feather growth. An experimental elevation of IGF-1 in adult bearded reedlings had no effects on feather growth, but increased moult intensity (Lendvai et al., 2021), whereas an experimental administration of IGF-1 to nestling pied flycatchers positively affected the growth of their tarsi but not of their wings (Lodjak et al., 2017). Hence, the present finding in adult Alpine swifts of a link between IGF1-1 and wing length is likely to be dependent on wing bones length rather than primary feathers length. A positive effect of IGF-1 on bone growth has been reported on numerous occasions [e.g., Yakar et al., 2002; Lodjak et al., 2016; Lendvai et al., 2021]. The effect of IGF-1 on bone growth appears, however, to be stronger on short than long bones, at least in laboratory models (Wang et al., 2006). It may explain why we only observed an association between IGF-1 with wing bones length (carpals and metacarpals) but not with sternum length, with the carpals and metacarpals being short boned whereas the sternum is one of the longest bones in swifts. The contrasting finding between IGF-1 and wing length in experimentally manipulated nestling pied flycatchers and adult swifts may come from differences between species in the influence of IGF-1 on wing bone growth, considering that Alpine swifts spend most of their lifetime aloft (Liechti et al., 2013). Hence, one hypothesis is that the positive association between IGF-1 and wing length in adult swifts comes from early-life allocation of resources into wing bone growth. This hypothesis also assumes that IGF-1 should be repeatable through the individual's life, from the nestling to adult stage, which has been previously reported (Roberts et al., 1990; Yuan et al., 2009). In this study, IGF-1 was found to vary with sampling date, and thus IGF-1 may potentially change during the breeding season. Yet, variation in IGF-1 levels among individuals can still be higher that those within individuals, making the trait flexible but repeatable. Many examples of this can be found in the literature regarding different physiological traits such as glucose levels (Montoya et al., 2018, 2020), antioxidant defenses (Récapet et al., 2019) or mitochondrial function (Stier et al., 2019). Further studies are now needed in swifts and other bird species to examine the repeatability through individual's life in IGF-1 and test for effects early in life of IGF-1 on the growth of short (e.g., carpals and metacarpals) and long (tarsi, sternum) bones.

Our results also show that IGF-1 and telomere length were negatively related in adult swifts. Studies in laboratory models and humans indicate that IGF-1 has a positive effect on telomerase activity (Bayne and Liu, 2005), suggesting that IGF-1 and telomere length could be positively correlated (e.g., Barbieri et al., 2009; Moverare-Skrtic et al., 2009; Yeap et al., 2019, but see Matsumoto et al., 2015; Matsumoto and Takahashi, 2016). However, the experimental disruption of IGF-1 signaling pathway in laboratory animals has been found to extend lifespan (Holzenberger et al., 2003; Yuan et al., 2009), and the few studies performed in free-living animals corroborate such an inverse correlation between IGF-1 and lifespan (Lewin et al., 2017; Lodjak and Mägi, 2017) or in our study with a proxy of lifespan such as telomere length (Sirman, 2019). So, if the link between IGF-1 and telomere length is not due to telomerase, an alternative mechanism may be oxidative stress. Interestingly, reduced IGF-1 signaling has also been associated with increased *in vivo* resistance to oxidative stress in laboratory models (Holzenberger et al., 2003). Furthermore, administration of IGF-1 in wild birds was found to lead to greater oxidative damage (Lendvai et al., 2020; Vágási et al., 2020) and higher activity of glutathione peroxidase (Lodjak and Mägi, 2017; Lendvai et al., 2020), the later possibly reflecting up-regulated antioxidant activity in response to oxidative stress (Lodjak and Mägi, 2017; Lendvai et al., 2020). Variation in oxidative stress and telomere length have been linked to the aging process, with individuals with enhanced resistance to oxidative stress and/or longer telomeres having higher annual survival or better fitness (Bize et al., 2009), and importantly exposure to oxidative stress is thought to accelerate telomere shortening (Reichert and Stier, 2017; but see Pérez-Rodríguez et al., 2019). Therefore, a negative association between IGF-1 and telomere length as reported here in adult swifts and other previous studies (Barbieri et al., 2009; Moverare-Skrtic et al., 2009; Yeap et al., 2019) may come from cascading effects of IGF-1 on oxidative stress, affecting in turn telomere dynamics through direct or indirect pathways (Casagrande and Hau, 2019), an idea that requires now further empirical examination.

In conclusion, negative associations of IGF-1 with wing and telomere length found here suggest that IGF-1 may play a role in regulating life-histories, and welcome future studies exploring the association of IGF-1 with direct measurements of growth and lifespan in wild organisms.

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 reviewed and approved by Swiss Federal Food Safety and Veterinary Office (FSVO).

AUTHOR CONTRIBUTIONS

BM, PB, and ÁL designed the study. BM, PB, and AS carried out the fieldwork. SZ and FC carried the laboratory analysis and interpretation of TL measures. ZT and ÁL carried the laboratory analysis and interpretation of IGF-1 measures. BM and PB carried out the data analyses. BM wrote the first draft. All authors critically revised the manuscript.

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Individual Variation in Thermal Reaction Norms Reveals Metabolic-Behavioral Relationships in an Ectotherm

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Ectothermic organisms respond to rapid environmental change through a combination of behavioral and physiological adjustments. As behavioral and physiological traits are often functionally linked, an effective ectotherm response to environmental perturbation will depend on the direction and magnitude of their association. The role of various modifiers in behavioral-physiological relationships remains largely unexplored. We applied a repeated-measures approach to examine the influence of body temperature and individual variation on the link between resting metabolic rate (RMR) and exploratory locomotor activity (ELA) in juvenile Alpine newts, *Ichthyosaura alpestris*. We analyzed trait relationships at two body temperatures separately and as parameters, intercepts and slopes, of thermal reaction norms for both traits. Body temperature affected the level of detectable among-individual variation in two different directions. Among-individual variation in ELA was detected at 12°C, while RMR was repeatable at 22°C. We found no support for a link between RMR and ELA at either temperature. While analysis of intercepts revealed among-individual variation in both traits, among-individual variation in slopes was detected in RMR only. Intercepts were positively associated at the individual, but not the whole-phenotypic, level. For ELA, the target of selection should be individual trait values across temperatures, rather than their thermal sensitivities. The positive association between intercepts of thermal reaction norms for ELA and RMR suggests that phenotypic selection acts on both traits in a correlated fashion. Measurements at one body temperature and within-individual variation hide the metabolic-behavioral relations. We conclude that correlative studies on flexible behavioral and physiological traits in ectotherms require repeated measurement at two or more body temperatures in order to avoid misleading results. This approach is needed to fully understand ectotherm responses to environmental change and its impact on their population dynamics.

Keywords: energy management, locomotor activity, metabolic rate, thermal adaptation, amphibians, repeatability

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INTRODUCTION

Ectothermic organisms respond to rapid environmental change through a combination of physiological and behavioral adjustments (Bennett and Huey, 1990; Angilletta et al., 2006; Williams et al., 2008; Huey et al., 2012). As these traits are often functionally linked (Careau et al., 2008; Biro and Stamps, 2010; Mathot and Dingemanse, 2015), an effective response to environmental change will depend on the magnitude and direction of their association. For example, metabolic rate fuels

functions and processes underlying locomotion, while locomotor activity is involved in both energy expenditure (e.g., mate searching) and energy gain (e.g., foraging) (Mathot and Dingemanse, 2015). Accordingly, locomotor activity level contributes to the acquisition and allocation of energy into maintenance, growth, reproduction, and survival, and thus to individual fitness in a given habitat (Burger et al., 2021). Information on behavioral and physiological covariation in a thermal context is required to understand the mechanistic link between individual traits and population dynamics under environmental change (Johnston et al., 2019); however, this issue has received surprisingly limited attention.

Current theory predicts that the relationship between metabolic rate and locomotor activity will depend on how an organism manages its energy budget (Careau and Garland, 2012; Mathot and Dingemanse, 2015). Under the "allocation model," which assumes a fixed daily energy expenditure, an individual with a high standard metabolic rate can invest less energy into locomotor activity than an individual with low maintenance costs, and *vice versa*. The "performance model," on the other hand, assumes a positive relationship (slope > 1; Halsey et al., 2019) between maintenance metabolic rate and daily energy budget. Under this scenario, locomotor activity should be positively related to standard metabolic rate, irrespective of its influence on the energy budget. Finally, the "independent model" assumes no relationship between maintenance metabolic rate and energy available for other behavioral (e.g., locomotor activity) and physiological tasks (e.g., digestion) under non-restricted total energy expenditure. In this case, a positive relationship is predicted between energy-gaining locomotor activity and maintenance metabolic rate because of a positive association between maintenance metabolic rate and daily energy expenditure (Mathot and Dingemanse, 2015).

A recent meta-analysis revealed no relationship between resting metabolic rate (RMR) and locomotor activity across taxa (Mathot et al., 2019), which was interpreted as being the result of interspecific variation in net energy contribution of this behavioral trait to the total energy budget. However, there are at least two alternative explanations for this result in ectotherms. First, that metabolic-behavioral relationships are affected by environmental stress (e.g., temperature), which may mask trait covariation due to individual variation in the slope or shape of their thermal reaction norms (Killen et al., 2013). Second, trait covariation may be attenuated by low repeatability (i.e., the relative amount of among-individual variation) of one or both traits at a given body temperature. Unfortunately, most studies on metabolic-activity relationships have been performed at one body temperature only and have used phenotypic correlations, which assume thermal independence of the link, perfect repeatability (R = 1) of both traits, and/or identical directions for among- and within-individual correlation. However, available studies have shown that the metabolic-activity link will change with temperature in an endotherm (Chappell et al., 2004), that the average repeatability of behavioral and physiological traits is 0.4 (Bell et al., 2009; White et al., 2013; Holtmann et al., 2017) and that metabolic-behavioral trait correlations vary at different variation levels (Gifford et al., 2014; Videlier et al., 2019), which suggests that temperature and within-individual (residual) variation may attenuate or mask trait correlation at the whole-phenotypic level. The empirical support for this prediction is missing (Dingemanse and Dochtermann, 2013; Killen et al., 2013; Careau and Wilson, 2017).

In this study, we undertook repeated measurements of RMR (energy consumption of post-absorptive and non-active, but immature, individuals during their typical inactivity period) and exploratory locomotor activity (ELA; distance covered in a new open-field arena) in juvenile newts at two body temperatures in the autumn and the following spring. While both traits are repeatable and thermally dependent in newts (Gvoždík and Kristín, 2017; Baškiera and Gvoždík, 2019, 2021), their covariation level is unknown. A recent analysis of metabolic traits has provided support for the allocation model of energy management in newts (Baškiera and Gvoždík, 2020), implying that energy-consuming ELA should be negatively associated with RMR. However, among-individual variation in the slopes of their thermal reaction norms for ELA is negligible (Baškiera and Gvoždík, 2020), suggesting that the masking or attenuating effect of its within individual variation modifies the RMR-ELA relationship between temperatures. To demonstrate the joint effect of body temperature and among-individual variation on the association of RMR and ELA, we first examined trait relationships at two body temperatures in addition to the commonly used "single temperature" approach. As a next step, we analyzed thermal reaction norm parameters for these traits with respect to among- and within-individual variation.

MATERIALS AND METHODS

Study Organisms and Maintenance

The alpine newt, *Ichthyosaura alpestris*, is a widespread European tailed amphibian with a body size up to 12 cm. Adults have an aquatic phase from April to June, and a terrestrial phase that lasts the rest of year. From November to April, the newts overwinter in underground shelters. Juveniles metamorphose in August and September and remain strictly terrestrial until maturity. Alpine newts are highly secretive and display mostly crepuscular and/or nocturnal activity. They feed on a wide range of invertebrate prey (Griffiths, 1996).

For this experiment, we used juvenile newts (n = 62) obtained from a large stock of newt larvae (clutches of 15 females) kept in outdoor tanks (n = 30) filled with non-chlorinated well water (90 × 63 × 47 cm; density = 30 ind. m^2) under semi-natural conditions (surface water temperature = 16.6 ± 6.0 [SD]°C; light intensity = 6.44 ± 18.13 × 10^3 lx). Haphazardly chosen metamorphs were weighed to the nearest 0.001 g (KERN EG, Balingen, Germany) and placed in plastic tanks (40 × 26 × 18 cm; n = 4 per tank) in walk-in environmental rooms with a fluctuating temperature regime (night minimum–day maximum = 12–22°C) and a naturally changing light:dark period (16:8–8:16; light intensity = 300 lx). This thermal regime was chosen to match the newts' preferred body temperatures (Gvoždík and Kristín, 2017) and mean air temperatures they normally experience in the field (Šamajová and Gvoždík, 2010). The bottom of each

tank was provided with moistened filter paper and a ceramic shelter. Every 3 days, the tanks were thoroughly washed and new substrate provided. The newts were fed with live *Tubifex* worms and chironomid larvae two or three times per week. Each newt was individually marked with a combination of implanted fluorescent elastomers (NorthWest Marine Technology, Shaw Island, United States). In November, we stopped feeding the newts and gradually reduced the air temperature to 4°C. Newts wintered under these conditions until the end of March. From April, the newts were exposed to the same temperature and light conditions as during the autumn.

Metabolic Rate Assays

Metabolic rate was measured indirectly as the rate of oxygen consumption, using flow-through intermittent respirometry. A more detailed description of the system, along with its verification and calibration, is provided elsewhere (Kristín and Gvoždík, 2012). In short, we flushed CO_2-free and water-saturated air (flow rate = 120 ± 1 ml min^{-1}) through each chamber (30 ml) of a nine-channel (eight chambers and one baseline) respirometry system (Sable Systems, Las Vegas, NV, United States) fitted with a baselining unit and an RM-8 multiplexer, twice per hour (enclosure time = 1,679 s) for a total of 5 h. The expired air was then passed through a Nafion^{TM} desiccator, a CO_2 analyzer, a scrubber, and an oxygen analyzer (FoxBox-C, Sable Systems). The respirometry chambers were placed in a water bath at 12 and 22 ± 0.5°C, while room temperature was maintained at 5 ± 2°C higher than the experimental temperature to prevent water condensation inside the respirometry system. The experimental temperatures were chosen to cover the newts' daily temperature range under rearing conditions.

We measured RMR at both temperatures in October and May, using the simplest repeated-measures design, i.e., two body temperatures with two repeated measurements (Killen et al., 2016), to reduce both newt habituation to experimental settings and the duration of measurement bouts. A 7-month time interval between measurement bouts was chosen to examine among-individual (co)variation before and after the cycle of acclimation to wintering temperatures and re-acclimation to thermal conditions during the following activity season. As newts are mostly crepuscular to nocturnal, all metabolic trials took place during the day (08:00–19:00). For each trial, the order of individuals and experimental temperatures was randomized. Newts were weighed to the nearest 0.001 g (KERN EG, Balingen, Germany) and individually placed into a respirometry chamber. Each newt was starved for 5 days prior to the metabolic trials to induce a post-absorptive state (Gvoždík and Kristín, 2017). Since locomotor activity greatly affects newt metabolic rate (Kristín and Gvoždík, 2012), newt activity was continuously monitored inside the chambers using web cameras connected to a PC with motion detection software (5 fps; iSpy software)[1] to ensure that newt activity was negligible during the measurement time interval. The lowest oxygen consumption (ml h^{-1}) of non-moving individuals (> 90% of enclosure time) from each trial was calculated from peak areas (integrals) of inverted raw oxygen measurements, divided by chamber enclosure time (Lighton, 2008; Kristín and Gvoždík, 2012). If locomotor activity violated standard conditions for RMR, they were discarded from further analyses. Data acquisition and calculations were performed using Expedata software v. 1.9.17 (Sable Systems).

Locomotor Activity Assays

All newts were rested for 2 days between RMR and ELA measurements. To measure ELA, circular acrylic arenas (140 × 10 mm; n = 9, illuminated from the underneath by IR light, were used to record newt activity at two body temperatures, 12 and 22°C in October and May. Unlike RMR measurements, which were performed in water-saturated air, newt body temperature during ELA trials can be affected by evaporative cooling. Accordingly, we estimated body temperature by measuring the operative temperature inside agar models of a similar size to the experimental individuals (Navas and Araujo, 2000). Visual communication between newts was prevented by placing opaque walls between each arena. Each trial consisted of a 5 min habituation period, followed by a 15 min recording period. After each trial, the experimental arenas were thoroughly cleaned with 95% ethanol to remove chemical cues from previously tested newts. Newts' position during the trial was continuously recorded using an automated tracking system (3.75 fps; Ethovision XT, Noldus, Wageningen, Netherlands), with ELA calculated as total distance (cm) covered during a trial. Software tracking was checked for missing values prior to calculation, and trials with > 5% missing samples were discarded from further analyses. All trials were performed in an environmental room without human presence during the newt activity period, i.e., after sunset between 20:00 and 22:00. We used satiated newts for each locomotor trial, with the order of individuals and the order of experimental temperatures randomized.

Statistical Analysis

We obtained information on RMR and ELA (co)variation using Bayesian multivariate mixed-effect modeling. First, we analyzed trait (co)variances at each body temperature separately. The model included month of measurement as a fixed factor, body mass as the linear covariate, and individual identity as a random factor. The variables examined and the covariate were mean-centered and divided by sample standard deviation to standardize variation to the same scale. Next, we analyzed (co)variances in the intercepts and slopes of individual thermal reaction norms. We examined (co)variation between intercepts and slopes for both traits, using the bivariate model with random intercept and slopes (Mitchell and Houslay, 2021). The fixed model structure included body temperature and body mass. The random structure consisted of two factors, individual identity and month, with random slopes.

Models were run with three expanded-parameter priors to evaluate the robustness of the results (**Supplementary Table 1** and **Figures 1–3**). Each model run consisted of 830,000 iterations, with the first 30,000 discarded. The remaining chain was sampled every 200th iteration, which resulted in a sample size of 4,000 estimates for posterior distribution. Each chain was

[1]http://www.ispyconnect.com

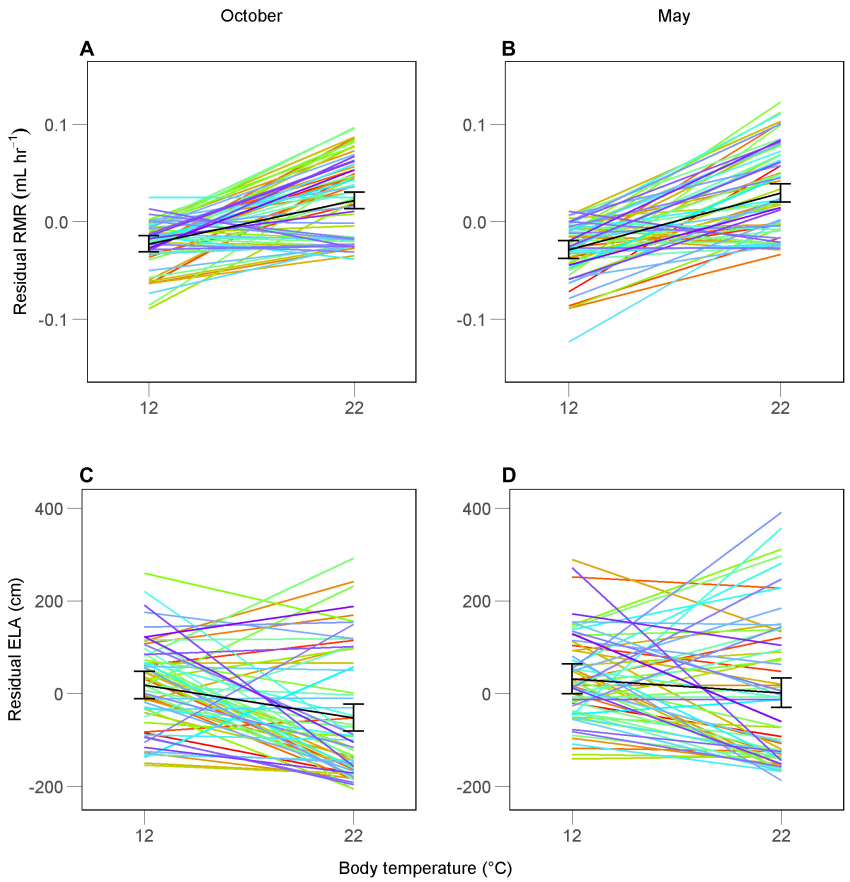

FIGURE 1 | Repeated measurements of mass-adjusted resting metabolic rate (RMR; **A,B**) and exploratory locomotor activity (ELA; **C,D**) in juvenile Alpine newts (*Ichthyosaura alpestris*) at two body temperatures between October and the following May. Each line shows values for one individual, while the black line shows the population mean (95% CI). Values were standardized to zero mean and divided by the sample standard deviation to allow variance comparison between traits.

tested for autocorrelation (< 0.1 in all cases) and convergence (Heidelberger and Gelman diagnostics). While all models with different priors produced similar parameter estimates, we chose the one with the best chain convergence for statistical inference. We considered any parameter in which the 95% credible interval (CI) did not include zero as statistically significant. For both variance and repeatability estimates (where values cannot be negative), we considered very low positive values ($< 10^{-7}$) as zero (Houslay and Wilson, 2017) and checked truncation in their posterior distribution. We then calculated variance explained by fixed model structure ($R_m{}^2$) and the whole model ($R_c{}^2$; Nakagawa and Schielzeth, 2013).

We calculated trait repeatability (R) and correlations (r) from their individual and residual variances (V) and covariances (Cov). Trait repeatability (intraclass correlation) for trait x was calculated as $R_\mathrm{x} = \frac{V_\mathrm{ind,x}}{V_\mathrm{ind,x}+V_\mathrm{e,x}}$, where $V_\mathrm{ind,x}$ is among-individual variation and $V_\mathrm{e,x}$ is residual variation. In the case of bivariate model with two random factors, we calculated trait repeatability as $R_\mathrm{x} = \frac{V_\mathrm{ind,x}}{V_\mathrm{ind,x}+V_\mathrm{month,x}+V_\mathrm{e,x}}$, where $V_\mathrm{month,x}$ is between-month variation (Araya-Ajoy et al., 2015). It represents the amount of among-individual variation due to permanent environmental effects and genetic differences. Finally, we used the formula after Araya-Ajoy et al. (2015) to calculate the repeatability of slopes of thermal reaction norms as $R_\mathrm{xslope} = \frac{V_\mathrm{ind,xslope}}{V_\mathrm{ind,xslope}+V_\mathrm{month,xslope}}$. Note that R_xslope is not directly comparable to R_x, because slopes and intercepts have different units, and thus the formula lacks residual variance in the denominator. Unlike R_x, this metric shows the amount of among-individual variation due to long-term consistency. Individual correlation (r_ind) was calculated as $r_\mathrm{ind} = \frac{Cov_\mathrm{ind,xy}}{\sqrt{V_\mathrm{ind,x} \times V_\mathrm{ind,y}}}$, where $Cov_\mathrm{ind,xy}$ is among-individual covariation between trait x and y. Similarly, residual correlation (r_e) was calculated as $r_\mathrm{e} = \frac{Cov_\mathrm{e,xy}}{\sqrt{V_\mathrm{e,x} \times V_\mathrm{e,y}}}$, where $Cov_\mathrm{e,xy}$ is residual covariation between trait x and y. Finally, phenotypic correlation (r_p) was calculated as $r_p = \frac{Cov_\mathrm{ind,xy}+Cov_\mathrm{e,xy}}{\sqrt{(V_\mathrm{ind,x}+V_\mathrm{e,x})\times(V_\mathrm{ind,y}+V_\mathrm{e,y})}}$ or $r_\mathrm{p} = \frac{Cov_\mathrm{ind,xy}+Cov_\mathrm{month,xy}+Cov_\mathrm{e,xy}}{\sqrt{(V_\mathrm{ind,x}+V_\mathrm{month,x}+V_\mathrm{e,x})\times(V_\mathrm{ind,y}+V_\mathrm{month,y}+V_\mathrm{e,y})}}$ in the case of model with two random factors. The statistical significance of R and r were inferred using the same approach as for model parameters. We used the best linear unbiased predictors (BLUP) obtained from multivariate mixed models to visualize trait associations at the individual level (Houslay and Wilson, 2017). All statistical

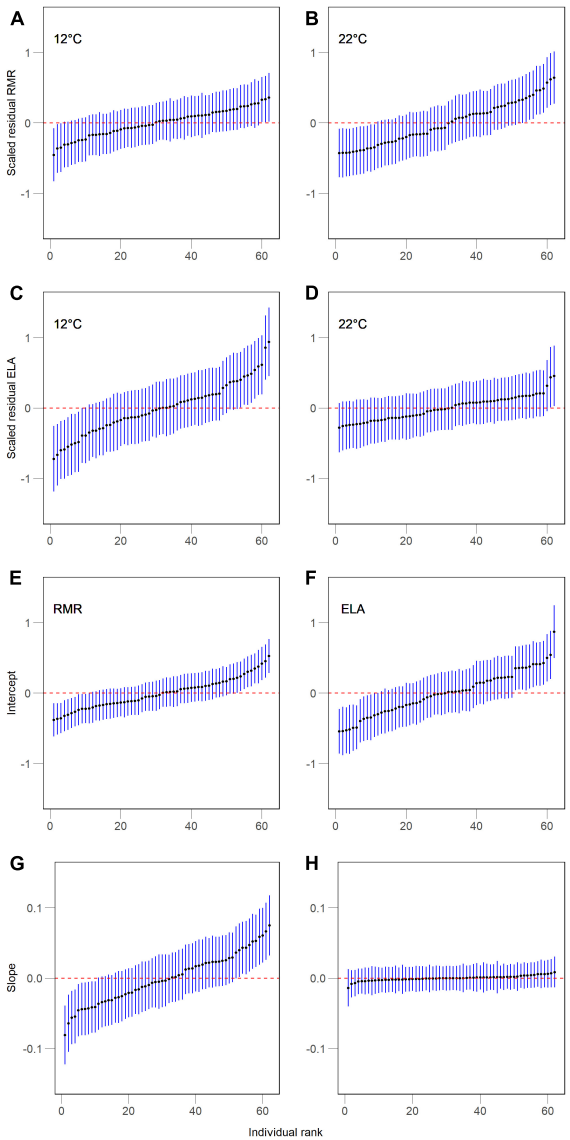

FIGURE 2 | Individual random intercepts of resting metabolic rate (RMR) and exploratory locomotor activity (ELA) at two body temperatures **(A–D)** and as parameters of their thermal reaction norms, intercepts and slopes **(E–H)** in juvenile Alpine newts (*Ichthyosaura alpestris*). Values are estimates (SE) of best linear unbiased predictors (BLUP) for each individual from their respective model (**Tables 1**, **3**).

calculations were performed in the R statistical environment (R Development Core Team, 2010, Vienna, Austria), using the "MCMCGlmm" (Hadfield, 2010) and "CODA" (Plummer et al., 2006) packages.

RESULTS

We obtained 496 repeated measures of RMR and ELA from 62 individuals (**Figures 1A,B**). Both traits were affected by body mass [October: 0.76 ± 0.12 (*SD*) g; May: 1.04 ± 0.24 g] at both body temperatures, with RMR increasing with body mass and ELA decreasing (**Table 1**). Mass-adjusted mean RMRs were similar between months at both body temperatures (**Figures 1A,B**). While mass-adjusted mean ELA showed similar values between months at 12°C, however, newts increased their average locomotor activity at 22°C in May (**Figures 1C,D**).

At 12°C, we detected repeatable among-individual variation in ELA, but not in RMR (**Table 2** and **Figures 2A,C**). Association between both traits was statistically non-significant at all variation levels (**Table 2** and **Figure 3A**). At 22°C, we found support for repeatability of RMR, but not ELA (**Table 2** and **Figures 2B,D**). Further, we found no evidence for trait association at this body temperature (**Table 2** and **Figure 3B**).

The fixed structure of random intercept and slope model showed that RMR increased with body mass, while ELA decreased (**Table 3**). Intercepts of thermal reaction norms for both traits and slopes for RMR contained detectable amounts of among-individual variation (**Table 3** and **Figures 2E–H**). There was no significant covariation between intercepts and slopes among individuals and between months in either trait (**Table 3**). Intercepts of thermal reaction norms for RMR and ELA were positively associated at the individual level, but not at the whole-phenotypic or residual levels (**Table 2** and **Figure 3C**). As the credible interval for r_{ind} was broad, we were unable to obtain any firm conclusion on the exact magnitude of this association. Slope values indicated a disparate effect of body temperature on RMR and ELA (**Table 3**), with positive values indicating that RMR increased with temperature (**Figures 1A,B**), while ELA decreased (**Figures 1C,D**). We found no support for the repeatability of slopes for ELA and its between-trait association at any variation level (**Table 3** and **Figure 3D**).

DISCUSSION

Despite their fundamental importance in behavioral and physiological ecology (Careau et al., 2008; Biro and Stamps, 2010; Careau and Garland, 2012; Mathot and Dingemanse, 2015; Killen et al., 2016), factors affecting metabolic-behavioral relationships remain poorly understood. We identified body temperature and among-individual trait variation (repeatability) as important modifiers of the relationship between RMR and ELA in juvenile newts and showed that detection of among-individual variation in RMR and ELA varies with body temperature. At 12°C, we only detected repeatability in ELA, while at 22°C, we detected repeatability in RMR but not ELA. Accordingly, we were only able to obtain poor estimates for their association at each body temperature. In contrast, intercepts of thermal reaction norms across these temperatures showed repeatability in both traits, with the traits being positively associated at the individual, but not the whole-phenotypic, level.

Our results provide evidence for a positive association between RMR and ELA in juvenile newts at the individual level. While a recent meta-analysis found no support for this relationship across taxa, which was interpreted as the result of the ambiguous impact of locomotor activity on individual

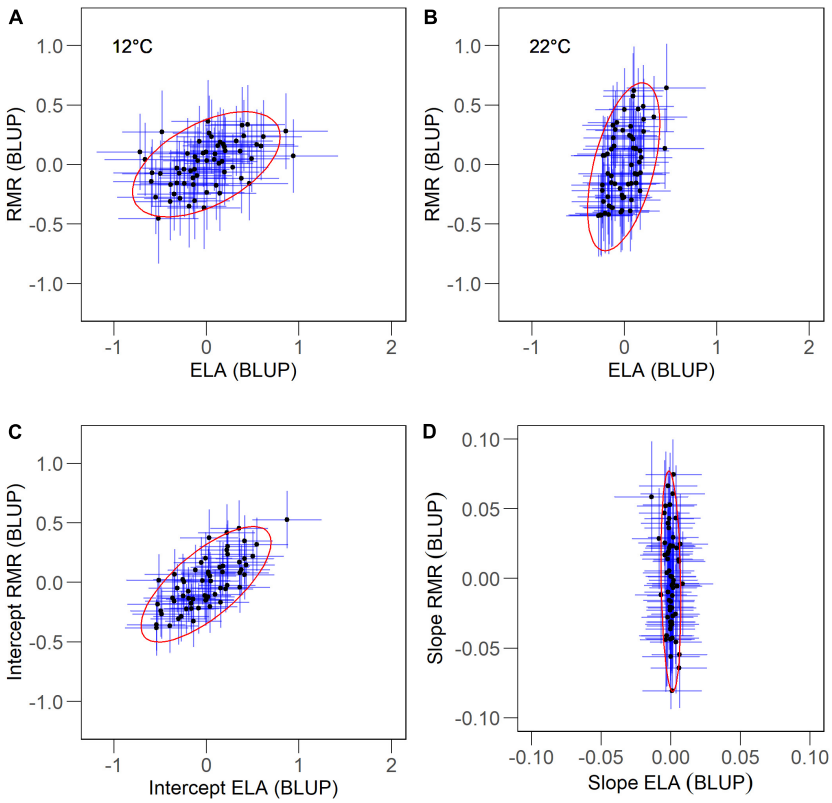

FIGURE 3 | Individual-level associations between resting metabolic rate (RMR) and exploratory locomotor activity (ELA) at two body temperatures **(A,B)** and as parameters of their thermal reaction norms, intercepts and slopes **(C,D)**, in juvenile Alpine newts (*Ichthyosaura alpestris*). Values are estimates (SE) of best linear unbiased predictors (BLUP) for each individual from the respective model (**Tables 1**, **3**). Values are fitted with 95% confidence ellipses.

energy budget (Mathot et al., 2019), our results suggest a previously unconsidered explanation, i.e., that the inconclusive link between metabolic rate and locomotor activity results from the opposing effect of body temperature on among-individual variation in each trait. In our study, the variation pattern produced poor estimates for correlation coefficients at both temperatures; however, "averaging" trait values across temperatures (intercepts of thermal reaction norm) stabilized individual rank for each trait, which improved estimates of individual correlation coefficient. This demonstrates the necessity to examine metabolic-behavioral relationships using repeated measurements from at least two body temperatures to obtain representative values for ectotherm taxa.

We observed that the relationship between RMR and ELA remained inconclusive at both body temperatures in juvenile newts. The body temperatures used here were within the range of preferred body temperatures for this species (Gvoždík and Kristín, 2017), suggesting that the newts in our study were not thermally stressed. According to a recent theory, the absence of thermal stress should not affect physio-behavioral associations (Killen et al., 2013). However, our results showed that even in the absence of stress, temperature changes altered the amount of among-individual variation in opposite directions for each trait, which resulted in similarly weak support for their association. At present, it is not known how thermal stress alters the association between metabolism and behavior in other ectotherms. While it is known that experimental temperature is an important modifier of the relationship between metabolism and behavior in endotherms (Chappell et al., 2004), the results are not directly comparable between endotherms and ectotherms due to the disparate influence of environmental temperature on their metabolic rate (McNab, 2002). Although further empirical studies are needed to fully understand the effects of environmental stressors on trait (co)variation, it is already clear that trait (co)variation is more sensitive to environmental changes than has previously been thought.

In our study, unlike intercepts, slopes contained significant among-individual variation in RMR only. Previously published results provide mixed support for this finding (Careau et al., 2014; Mitchell and Biro, 2017; Baškiera and Gvoždík, 2019; Kar et al., 2021), suggesting that the amount of among-individual variation in thermal sensitivity varies substantially among taxa and traits. Among-individual variation in slopes appears to be affected by the disparate effects of temperature on physiological and behavioral traits (Abram et al., 2017). The thermal dependence observed in RMR is a typical example of a kinetic effect, which is determined by the amount of kinetic energy in a physiological

TABLE 1 | Parameters [estimates with 95% credibility intervals (CI)] of multivariate mixed models examining co(variation) between resting metabolic rate (RMR) and exploratory locomotor activity (ELA) in juvenile Alpine newts (*Ichthyosaura alpestris*) at two body temperatures.

Factors	12°C	22°C
FIXED	β	β
RMR		
Intercept	0.09 (−0.16, 0.33)	−0.01 (−0.24, 0.22)
Body mass	**0.56** (0.39, 0.77)	**0.63** (0.44, 0.80)
Month	−0.17 (−0.53, 0.18)	0.03 (−0.28, 0.37)
ELA		
Intercept	0.17 (−0.11, 0.44)	0.23 (−0.06, 0.50)
Body mass	**−0.40** (−0.62, −0.17)	−0.22 (−0.45, 4.53×10^{-3})
Month	−0.35 (−0.70, 0.06)	**−0.45** (−0.87, −0.05)
RANDOM	δ^2	δ^2
Among-individual		
V_{RMR}	**0.13** (1.03×10^{-6}, 0.30)	**0.21** (2.46×10^{-2}, 0.18)
V_{ELA}	**0.31** (2.04×10^{-2}, 0.57)	0.14 (6.61×10^{-9}, 0.36)
$Cov_{RMR,ELA}$	0.06 (-6.31×10^{-2}, 0.19)	0.05 (-6.07×10^{-2}, 0.18)
Within-individual		
V_{RMR}	**0.54** (0.37, 0.75)	**0.43** (0.28, 0.61)
V_{ELA}	**0.61** (0.39, 0.84)	**0.88** (0.59, 1.16)
$Cov_{RMR,ELA}$	0.03 (−0.12, 0.18)	0.05 (−0.09, 0.21)
	R_m^2 = 0.13, R_c^2 = 0.42	R_m^2 = 0.11, R_c^2 = 0.28

Statistically significant values are in bold. R_m^2, variance explained by fixed factors; R_c^2, variance explained by the whole model.

TABLE 2 | Repeatability of resting metabolic rate (RMR) and exploratory locomotor activity (ELA) and their correlation coefficients measured at two body temperatures and as intercepts of their reaction norms in juvenile Alpine newts (*Ichthyosaura alpestris*).

Trait	12°C		22°C		Intercept	
	Estimate	95% CIs	Estimate	95% CIs	Estimate	95% CIs
Repeatability						
RMR	0.19	0, 0.40	**0.32**	0.06, 0.55	**0.24**	0.05, 0.41
ELA	**0.32**	0.07, 0.56	0.13	0, 0.33	**0.25**	0.05, 0.43
Correlation						
Phenotypic	0.07	−0.07, 0.27	0.15	−0.05, 0.29	0.14	−0.03, 0.29
Individual	0.30	−0.41, 0.92	0.50	−0.46, 0.98	**0.50**	0.03, 0.93
Residual	0.08	−0.20, 0.28	0.11	−0.15, 0.30	0.15	−0.15, 0.44

Statistically significant values (i.e., 95% credibility intervals excluding zero) are in bold.

system. In contrast, ELA is mostly affected by the integrated effect of temperature, which results from sensed thermal information integrated by the neural system. Accordingly, the shape of reaction norms for ELA varies between newt life stages and differs substantially from thermal performance curves for forced locomotion (Baškiera and Gvoždík, 2019; Gvoždík and Boukal, 2021). Given the presence of among-individual variation is the basic assumption for evolution by natural selection, different amount of this variation in slopes for RMR and ELA suggests that thermal sensitivities of physiological and behavioral traits evolve in disparate rates in newts (Gvoždík, 2015) as in other long-lived ectotherms (Muñoz et al., 2014; Bodensteiner et al., 2021).

One could argue that our estimates of among-individual variation are confounded by the experimental design used. Thermal reaction norms, for example, are often non-linear (Huey and Stevenson, 1979; Amarasekare, 2015;

TABLE 3 | Parameters [estimates with 95% credibility intervals (CI)] for multivariate mixed models examining co(variation) among intercepts and slopes of reaction norms for resting metabolic rate (RMR) and exploratory locomotor activity (ELA) in juvenile Alpine newts (*Ichthyosaura alpestris*).

Fixed factors	β	Random factors	Among-individual δ^2	Between-month δ^2	Within-individual δ^2
RMR		V_{RMRint}	**9.56×10^{-2}** (2×10^{-2}, 0.18)	**0.11** (2.36×10^{-2}, 0.21)	**0.19** (0.11, 0.29)
Intercept	1.36×10^{-3} (−0.11, 0.11)	$V_{RMRslope}$	**2.71×10^{-3}** (2.45×10^{-4}, 0.04×10^{-2})	0.21×10^{-2} (1.59×10^{-10}, 0.42×10^{-2})	
Body mass	**0.46** (0.37, 0.56)	V_{ELAint}	**0.20** (2.06×10^{-2}, 0.37)	**0.34** (1.63×10^{-1}, 0.50)	**0.27** (0.13, 0.41)
Temperature	**0.10** (0.08, 0.12)	$V_{ELAslope}$	3.91×10^{-4} (1.50×10^{-14}, 0.01×10^{-2})	6.71×10^{-2} (2.91×10^{-3}, 1.07×10^{-2})	
ELA					
Intercept	1.16×10^{-3} (−0.17, 0.16)	$Cov_{RMRint,RMRslope}$	9.74×10^{-3} (-6.04×10^{-4}, 0.02)	1.09×10^{-2} (-3.09×10^{-4}, 0.02)	
Body mass	**−0.16** (−0.30, −0.03)	$Cov_{ELAint,ELAslope}$	9.16×10^{-4} (-4.82×10^{-2}, 0.20)	3.44×10^{-2} (-1.83×10^{-2}, 0.05)	
Temperature	**−0.04** (−0.06, −0.02)	$Cov_{RMRint,ELAslope}$	-1.57×10^{-4} (-6.62×10^{-2}, 5.22×10^{-2})	2.65×10^{-2} (-8.09×10^{-3}, 0.01)	
		$Cov_{ELAint,RMRslope}$	5.82×10^{-3} (-5.83×10^{-3}, 0.02)	-1.21×10^{-2} (-1.34×10^{-2}, 0.01)	
		$Cov_{RMRint,ELAint}$	6.74×10^{-2} (-3.24×10^{-3}, 0.15)	-1.97×10^{-2} (-2.44×10^{-2}, 0.22)	0.03 (−0.04, 0.11)
R_m^2 = 0.17, R_c^2 = 0.70		$Cov_{RMRslope,ELAslope}$	-2.25×10^{-5} (-1.01×10^{-3}, 9.82×10^{-2})	6.71×10^{-4} (-1.20×10^{-3}, 2.45×10^{-2})	

Statistically significant values are in bold.

Little and Seebacher, 2021), whereas our measurements at two body temperatures assume a linear thermal dependence of the traits studied. Although the thermal dependence of ELA is U-shaped across the broad body temperature gradient, both ELA and RMR are linear within the limited temperature range used in the present study (Gvoždík and Kristín, 2017; Baškiera and Gvoždík, 2019). Accordingly, our "two-temperature" approach provided unbiased estimates of the variation in the slopes of thermal reaction norms. Another source of bias could arise from neglecting the maternal effect on examined traits (Bernardo, 1996; Mousseau and Fox, 1998; Uller, 2008). For logistical reasons, we could not raise newts from each clutch separately, and therefore consider maternal identity as an additional random factor. Therefore, we cannot exclude the possibility that among-individual variation in the traits studied was overestimated compared to values obtained from randomly selected individuals in a large population. Despite this shortcoming, the phenotypic similarity between offspring should not affect the main result of our study, i.e., the differential effect of body temperature on among-individual variation in physiological and behavioral traits.

The positive association between RMR and ELA has both ecological and evolutionary implications. From an ecological point of view, the positive RMR-ELA link suggests that juvenile newts manage their energy budget according to the performance model (Careau and Garland, 2012; Mathot and Dingemanse, 2015). This is contrary to recent findings showing a negative relationship between maintenance metabolic rate and energy available for other tasks, but is in accordance with the positive association between maintenance and maximum metabolic rate, in adult newts across body temperatures (Baškiera and Gvoždík, 2020). The likely explanation for this discrepancy is the context-dependency of energy management (Halsey et al., 2019). Juvenile newts, for example, show variation in their RMR and growth rates in the presence of intra- and interspecific competition (Janča and Gvoždík, 2017), which may corroborate this view. However, further experimental support is needed for a definite conclusion. From an evolutionary point of view, this finding suggests that phenotypic selection should act on RMR and ELA in a correlative manner. Theory suggests that the adaptive significance of RMR is highly context-dependent (Careau et al., 2008; Burton et al., 2011; Norin and Metcalfe, 2019). Positive RMR-ELA covariation means that if resources are widely available, selection should favor metabolically fast individuals with high locomotor activity level, while low metabolic rate and locomotor activity should be beneficial under a scarcity of resources. In ectotherms, context-dependent adaptive significance is complicated by variation in the thermal dependency of both traits. For example, under limited resources, high body temperatures disproportionally increase mandatory metabolic costs relative to locomotor activity level, which provides a further penalization to metabolically-fast individuals. As such, climate warming is likely to erode metabolic-behavioral variation within ectotherm populations in the near future.

To sum up, we demonstrated that body temperature and individual variation hide metabolic-behavioral relationships in an ectotherm, with body temperature modifying the link not through disparate thermal dependency of mean trait values but the amount of among-individual variation. By focusing on parameters of reaction norms rather than trait values at a single body temperature, we were able to identify the link between metabolic rate and locomotor activity. This clearly indicates the importance of a repeated measures design across at least two body temperatures for obtaining meaningful information on metabolic-behavioral links in ectotherms (Killen et al., 2016). The minimalistic approach could be further improved by adding more temperatures and repeated measures to increase the accuracy of parameter estimates (van de Pol, 2012; Dingemanse and Dochtermann, 2013; Mitchell and Houslay, 2021); however, finding an optimal solution among the statistical needs, logistical issues, and confounding effects of habituation or acclimation provides a challenging task in this field. Despite these issues, we believe that adopting the repeated measurements approach over two or more temperatures will substantially improve our understanding of the role of energy budget (Careau and Garland, 2012), pace of life syndrome (Goulet et al., 2017), and trade-offs (Careau and Wilson, 2017) in shaping ectotherm responses to environmental change.

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://doi.org/10.6084/m9.figshare.17013449.v1.

ETHICS STATEMENT

All experimental procedures were approved by the Expert Committee for Animal Conservation of the Institute of Vertebrate Biology of the Czech Academy of Sciences (research protocol no. 135/2016). Permission to capture newts was provided by the Agency for Nature Conservation and Landscape Protection of the Czech Republic (1154/ZV/2008).

AUTHOR CONTRIBUTIONS

LG conceived the ideas, designed the methodology, and analyzed the data. SB and LG collected the data and wrote the manuscript. Both authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.850941/full#supplementary-material

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Allostatic Load in Gambel's White Crowned Sparrow, *Zonotrichia leucophrys gambelii*: Relationships With Glucocorticoids

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Regulation of energetic expenditure in a changing environment, considered here as allostatic load, is central to organism-environment interactions. The value of responses that modify behavior or physiology in coping strategies is often measured in terms of energetic benefits. In this study, the total energetic cost incurred by Gambel's white-crowned sparrows, *Zonotrichia leucophrys gambelii*, was assessed using heart-rate transmitters. The use of heart rate was validated as a proxy for metabolic rate *via* flow-through respirometry. Applying heart rate as an indicator of allostatic load, we confirmed that ambient temperature under wintering conditions influences allostatic load. However, baseline corticosterone, proposed to mediate physiological responses to variation in allostatic load, does not appear to vary with heart rate or temperature in captivity, or with temperature under ambient conditions in the field. The relationship between allostatic load and plasma corticosterone levels was also investigated by manipulating feeding effort for captive Gambel's white-crowned sparrows using a sand-excavation challenge that approximated a type of foraging work that these birds normally perform in the wild. This experiment was designed to test the hypothesis that experimentally increased allostatic load induces elevation in baseline corticosteroids. We did not find support for this hypothesis. We suggest that the adrenocortical response to increased allostatic load may be limited to overload or environmental conditions that meaningfully threaten energy imbalance, indicating new targets for further research.

Keywords: allostatic load, glucocorticoid, heart rate, energy expenditure, oxygen consumption, temperature

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INTRODUCTION

The model of allostatic load outlined by McEwen and Wingfield (2003) emphasizes variation in energetic demand as a key driving force underlying glucocorticoid regulation of physiological and behavioral adaptations to a changing environment. Allostatic load, in this model, refers to the cumulative cost of allostasis, maintaining stability through change. Energy expenditure varies both predictably and unpredictably over the life of an animal. It is important for an individual to maintain neutral energy balance whenever possible despite fluctuations in demand, resources available and body condition (e.g., Broggi et al., 2019). Environmental factors are expected to be a primary source of variation in energy requirements, particularly in wintering animals (Wingfield and Ramenofsky, 2011; Broggi et al., 2019), when costs and trade-offs associated with breeding, molt, and migration are absent. When environmental factors create a psychological or

physiological perturbation, it can activate the emergency life-history stage (ELHS, Wingfield et al., 1998), which consists of a series of behavioral and physiological responses, regulated primarily by glucocorticoids, that mobilize resources and promote individual survival at the expense of other life-history strategies (Romero and Wingfield, 2016). The observation of organismal responses to fluctuations in winter weather offers an excellent opportunity to examine the overall energy demands an individual experiences at any moment that is a function of resources available in the environment, body condition, social status, etc. (Korte et al., 2005).

The model of allostatic load (McEwen and Wingfield, 2003) makes two independent assumptions that may be drawn upon for their testable predictions and alternatives. Assumption 1 states that costly events cause allostatic load to increase, at least up to a threshold for ELHS activation, which triggers compensatory mechanisms to reduce allostatic load. Alternatively, costly events could directly result in minor compensatory changes that reduce existence energy or routine energetic expenditures (e.g., by lower body temperature or reduced non-feeding activity) that keep allostatic load stable until such options are exhausted. This alternative would maximize the perturbation resistance potential (PRP), i.e., the difference between allostatic load and the cumulative resources available internally and in the environment (Wingfield et al., 2011, 2017).

Assumption 2 states that increased allostatic load is associated with elevation in circulating baseline glucocorticoid levels, at least up to a threshold for the ELHS.

Alternatively, if allostatic load changes (Assumption 1), plasma glucocorticoids may not change in parallel. Because the role of glucocorticoids during the ELHS transition is well established, sudden sustained or repeated increases may trigger the ELHS only when allostatic load approaches the limit of energy availability rather than gradually rising to a threshold. This alternative would link glucocorticoid action to PRP as opposed to allostatic load.

Heart rate is a common proxy for estimating metabolic rate in the field (reviewed in Butler et al., 2004; Green, 2011). While, heart rate (f_H) and oxygen consumption are generally correlated, unpredicted changes in stroke volume and/or oxygen extraction can result in estimation errors (particularly when one is used to predict the other). Although f_H and oxygen consumption are related, the consistency of this relationship is unclear. Relationships have been noted within individuals across seasons, as well as across individuals and species. Variation in the relationship can be related to body mass (BM; Green, 2011), but allometric scaling does not always account for observed differences (e.g., Portugal et al., 2009). The selection of appropriate scaling indices is, in itself, problematic (Packard and Boardman, 1999). In mammals, heart rate typically scales with mass ($BM^{-0.26}$) meaning f_H decreases as BM increases (White and Kearney, 2014). On the other hand, mammalian $\dot{V}O_2$, volume of oxygen consumed per unit time, increases with mass ($BM^{0.71}$) though the allometric scaling relationship of $\dot{V}O_2$ can vary with circumstance (White and Kearney, 2014). Procedures for handling scaling are diverse, including modeling body mass as a covariate, calculating mass exponents using a subset of data taken under constant conditions, or trying a range of possible mass exponents and selecting a value that explains the most variability.

Here we first performed experiments on captive, adult Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to validate the use of heart rate telemetry as a proxy for energy expenditure through simultaneous respirometry to evaluate the extent that these approaches reliably co-varied. We then evaluated intra- and inter-individual variations in f_H and explored a role for body mass in explaining this variability. Next, we employed this technique and others to investigate McEwen and Wingfield's (2003) model of allostatic load.

To evaluate Assumption 1, we determined whether typical fluctuations in outdoor temperatures during winter in Davis, California influenced allostatic load among captive and free-living birds. Ambient temperatures were usually below the laboratory-determined thermoneutral zone for this species (King, 1964). Allostatic load, in this case, was averaged over a 24-h period, and measured using f_H. Real-time heart rate data has the advantage of offering insight into how ambient temperature influenced f_H and to what extent compensation occurred within a 24-h average. Free-living birds experienced complex meteorological events and exploited microhabitat variation whereas captive birds were housed in sheltered outdoor aviaries with limited exposure to extraneous variables (e.g., wind) but experienced natural variation in ambient temperature and humidity. To evaluate Assumption 2, we compared heart rate telemetry data to measurements of baseline plasma corticosterone (the major glucocorticoid in birds). We also examined ambient temperature for relationships with baseline corticosterone on the basis of Assumption 1.

In the second part of this study, we experimentally manipulated energy expenditure to increase allostatic load to test for its impact on baseline plasma levels of corticosterone independent of ambient temperature thereby further evaluating Assumption 2. Energy expenditure has been manipulated in several species in captivity. Techniques previously applied to adult birds include perch removal, lever pressing, weight-carrying and motion-sensing feeders with pre-programmed frequency or probability of food release used to manipulate the effort required for food acquisition (Bautista et al., 1998; Wiersma et al., 2005; Johns et al., 2018). Physiological costs associated with normal seasonal changes of life-history stages, e.g., egg laying, have also been examined (Vézina et al., 2006). However, energetic demand associated with physiological change is problematic as a mode of "manipulation" because costs may change concurrently with a suite of allostatic shifts across life-history stage transitions potentially including changes at multiple levels of the hypothalamic-pituitary-adrenal (HPA) axis. For example, glucocorticoid receptor expression can change seasonally, which complicates interpretation of concurrent hormonal shifts (Lattin and Romero, 2014; Krause et al., 2015; Wingfield, 2018). Methods that involve manipulation of effort within season offer superior control of internal confounds. However, the unrealistic nature of challenges like perch removal or unpredictable food release, relative to a songbird's evolutionary history and life experience, may confound an energetic manipulation with a cognitive cue

that may act as a classical "stressor." This is particularly important in teasing apart the predictions of allostatic load from our understanding of the classical stress response.

The aim here was to experimentally increase workload for captive Gambel's white-crowned sparrows to mimic variation in foraging effort required in the field. Substrate-scratching is a mode of foraging behavior described in a variety of songbirds (Greenlaw, 1977). A study of free-living New World sparrows showed that increasing the amount of litter on top of food increases the frequency of engagement in prolonged feeding bouts, as measured by number of "scratches" per bout in those areas (Hailman, 1984).

Animals may also compensate for increased workload by diminishing energetic investment in other activities or functions. This issue is minimized in a captive setting where other energetic demands are absent. Heart rate telemetry could not be used in the second set of experiments due to problems using our transmitters inside small cages. Therefore, the verification of Assumption 2 relies heavily on the validity of Assumption 1, i.e., experimental workload can be applied as a direct proxy for allostatic load. To probe the validity of Assumption 1 in this context, we collected and analyzed 24 h video recordings to determine whether patterns of daytime energy expenditure changed as a result of foraging workload manipulation. We also measured food consumption and change in body mass as further indicators of energetic status. To evaluate Assumption 2, we tested baseline plasma corticosterone levels during a manipulated workload regime and with respect to activity level and consumption metrics within and across treatment regimes. Exploration of these outcomes has implications for understanding not only relationships between allostatic load and corticosterone, but also for individual variation.

MATERIALS AND METHODS

General Methods Applied Across Studies

Bird Capture and Housing

Gambel's white-crowned sparrows (hereafter, GWCS; *Z. l. gambelii*) winter in large numbers at our study sites in and around Davis, CA, United States (38.5°N, 121.8°W, **Supplementary Figure 1**, Chilton et al., 1995). We captured all birds in the winter months (November to February) using mist nets or Potter traps that were pre-baited with seeds (under guidelines of the State of California and USFWS collecting permits). All procedures and housing of captive birds were approved by the University of California Davis IACUC. For each bird, we measured morphological characteristics including length of the tarsus, beak (nare to tip) and flattened wing chord to the nearest 0.1 mm using calipers. We weighed birds (±0.1 g) using a Pesola scale.

All GWCS were initially housed in flight cages (1.8 m × 1 m × 1.8 m), typically within an outdoor aviary at UC Davis. The aviary was semi-enclosed, open to the outside air and naturally lit, but was sheltered from rain and prevailing winds by a roof and concrete walls on two sides. While in flight cages, birds were fed a diet consisting of Mazuri small bird maintenance feed (PMI Nutrition), wild bird seed, and vitamin-enriched grit *ab libitum*. We provided water in bottles as well as in cups with the latter mostly used by the birds for bathing. Birds were allowed to acclimate to captivity for at least 3 weeks prior to the initiation of experiments or transfer to individual cages (Wingfield et al., 1982).

Blood Sampling

We collected a small blood sample by puncturing the alar vein within 3 min of capture using a 26-gauge needle. We collected blood into heparin-coated microhematocrit tubes. Blood samples were briefly stored on ice, then centrifuged at approximately 170 *G* for 5 min. Plasma was aspirated and stored at −35°C until used for hormone assays. Baseline corticosterone samples from wintering GWCS were collected at multiple sites in Davis, CA between 2007 and 2014.

Corticosterone Assay

Circulating corticosterone (ng/mL) was measured by radioimmunoassay following Wingfield et al. (1992). Briefly, plasma was added in volumes ranging from 5 to 30 μL in combination with distilled water to achieve a consistent volume of 200 μL. 2,000 CPM of tritiated corticosterone (Perkin Elmer NET399250UC) was added to each sample to evaluate extraction efficiency of individual samples. After overnight equilibration, steroids were extracted with 4 mL of freshly re-distilled dichloromethane for 2 h. The organic phase was aspirated and dried under a stream of nitrogen gas in a water bath at 35°C, then reconstituted in 550 μL of phosphate-buffered saline with gelatin (PBSG). A 100 μL aliquot was removed and scintillation fluid added (Perkin Elmer Ultima Gold: 6013329) to evaluate percent recovery of the labeled hormone for each sample. Duplicate 200 μL aliquots were equilibrated overnight at 4°C with 100 μL (approx. 10,000 CPM) of tritiated corticosterone and 100 μL of antibody (100× dilution of Esoterix Inc. B3-163). Unbound steroid was stripped from solution by adding 500 μL of dextran-coated charcoal for 10 min at 4°C, followed by refrigerated centrifugation at 3000 RPM. Supernatant (containing antibody-bound hormone) was decanted and vortexed with scintillation fluid before counting for 10 min or within 2% accuracy on a Beckman 6500 liquid scintillation counter. Each assay included two solvent blanks and a standard sample (1,000 pg of unlabeled corticosterone). The mean recovery efficiency was 87.4% and coefficients of inter- and intra-assay variation were 16.1 and 12.9%, respectively. The mean lower detection limit was 18.05 pg/tube.

Heart Rate Telemetry and Signal Processing

SP2000 heart rate transmitters (Sparrow Systems, IL, United States) were attached using a protocol modified from Raim (1978). Each transmitter was attached to a small piece of cotton cloth (approximately 1.5 cm × 2.5 cm). Birds were briefly immobilized by isoflurane anesthesia. A small patch of contour feathers was trimmed (captive birds) or plucked (necessary for superior adhesion in field studies) from the dorsum, and two sterilized electrocardiogram (EKG) electrodes were inserted

subcutaneously (3–4 mm) *via* two small incisions (1 mm) lateral to the dorsal midline and just posterior to the scapula. Incisions were closed with surgical adhesive and treated with lidocaine. The cloth holding the transmitter was glued to the dorsum using latex eyelash adhesive (Duo brand), with the remaining length of the electrodes tucked under the cloth.

Heart rate signals were received using an AR8200 receiver (AOR Ltd., Tokyo, JP) with a telescopic whip (captive) or handheld Yagi (field) antenna. Audio output was recorded to an R09-HR recorder in MP3 format or, in the case of validation studies, directly to disk in WAV format using Audition software (Adobe Systems, Inc., CA, United States).

Heart rate data were extracted and filtered from audio files using Vireo software, two scripts written for Scilab (Versailles, France) by Kelley and Bisson (2009), with modifications to accommodate a range of heart rate and noise characteristics specific to each experiment. Extraction and filtration were spot-checked manually by removing the carrier frequency using an FFT filter in Adobe Audition software (Adobe Systems, Inc., CA, United States) and hand-counting peaks associated with heart beats. Data were extracted at a resolution of 1 datum point per second, then averaged over 5-min periods to reduce autocorrelation and accommodate any time offsets relative to $\dot{V}O_2$.

Validation of Heart Rate as a Proxy for Allostatic Load

Respirometry

Rates of O_2 consumption ($\dot{V}O_2$) of 7 captive adult GWCS (mean mass ± SEM: 30.18 ± 0.29 g; see **Supplementary Tables 1**, **2** for statistical analyses of mensural characters) were measured using open-flow respirometry. Room air was drawn at a rate of 900 mL/min from the bottom of a clear acrylic chamber (6L volume) fully air-tight except for an in-flow port at the top of the chamber (**Supplementary Figure 2**).

The respirometry chamber was equipped with food, water and a perch, just large enough for a bird to extend its wings, though too small to permit flight. A 6-cm fan was installed in the lid to prevent formation of dead spaces around the objects in the chamber. Pump, flow control, oxygen and carbon dioxide meters were included in a Foxbox (Sable Systems, Las Vegas, NV, United States). Water and CO_2 were removed from air prior to oxygen measurement using a laboratory gas drying unit (2 5/8 × 11 3/8, Hammond Drierite Co., OH, United States) and soda lime in a smaller (3/5 × 8″) column. To correct for drift in the instruments, a multiplex unit was engaged for automatic base-lining with room air for 1-min every 10-min. The chamber was placed in the room where birds were normally housed, and each subject was able to view conspecifics throughout. All other equipment was in an adjacent room, which allowed the experimenter to remain hidden from the subject's view during testing. Temperature in the bird room and adjacent to the Foxbox was measured using Sable thermistor probes connected to a universal interface (UI-2, Sable Systems). Respirometry data from the Foxbox were imported to a PC running Expedata software (v. 1.2.5, Sable Systems, Las Vegas, NV, United States). Oxygen measurements were corrected to STP (standard temperature and pressure) which were adjusted to baseline. Instantaneous $\dot{V}O_2$ was calculated using the manufacturer's recommended procedures for the Expedata software. Data were averaged over 5 min periods to align with heart rate.

We validated the stability of measurements in the apparatus using nitrogen injection (Lighton, 2008). Briefly, a known rate of oxygen "consumption" is generated *via* displacement of oxygen using a small known flow-rate of pure nitrogen gas added to the air intake. This known rate of change is then compared with instantaneous $\dot{V}O_2$ calculated based on oxygen measurement to verify that the respirometry chamber is not a source of error.

Heart Rate Telemetry, Validation Protocol

After we attached the heart rate transmitters, birds were returned to their cages and allowed to recover from anesthesia for at least 1 h. They were then placed in the respirometry chamber and allowed to settle while the respirometry system equilibrated. Validations began immediately upon placing the bird in the chamber and continued for a period of 2 h. To obtain maximal heart rate elevation birds were intermittently disturbed by the experimenter entering the room and briefly tapping on the chamber. All experiments were conducted at the end of the day (15:00–17:00). Overhead fluorescent lights were turned off 20 min prior to the end of each trial to allow the bird to settle down for the night. This range of activity revealed heart rates, approximately 440–900 beats per minute (BPM), which were nearly comparable to that of a free-living bird. While frequent technical challenges limited the validation period to 2 h there were opportunities to collect additional data over the course of the one or more days spent in the respirometry chamber for several birds. These data were examined to evaluate intra-individual variability and possible sources of error.

Validation Comparison: Selection and Conversion of Existing Data

To compare our f_H-$\dot{V}O_2$ validation data with existing data from other species, we performed a literature search to extract data for visual meta-analysis. These data included juvenile and adult birds from different species. Where findings did not support a single relationship across individuals (e.g., Gessaman, 1980), we selected individual relationships with minimum and maximum slopes separately for comparison. Because body mass varied widely across the data set, $\dot{V}O_2$ was converted to reflect units of ml/min/kg$^{0.703}$ (Tieleman and Williams, 2000) and heart rate was converted to BPM/kg$^{-0.26}$ (White and Kearney, 2014).

Measurement of Allostatic Load *via* Heart Rate Telemetry

Aviary Telemetry

Adult GWCS were captured during February of 2011 and 2012, processed and acclimated as described above. In the aviary, ambient temperature (°C) was monitored continuously using two thermistors taped to the walls of the cage that recorded to a PC running Expedata software (Sable Systems, Las Vegas, NV, United States). We used an average of the temperature readings (°C) from these two thermistors in our analyses. Transmitters

were attached to 5 birds for each of three trial periods. The AR8200 receiver was programmed to cycle through each of the 5 transmitter frequencies at a rate of 1 channel per minute. Signals for each individual were recorded for 1 min out of every 5-min period. Heart rate was recorded over a period of 7 days or until the transmitters failed, after which they were promptly removed from the birds. Audio files were split into 1 min segments for analysis. Within each minute, the first and last 10-s were eliminated to prevent error due to channel switching. Heart rates were averaged over 30-min periods (6 × 1-min samples for each of the 5 birds) to reduce autocorrelation and minimize the influence of brief disturbances.

Heart rate was measured for 5 birds starting on March 18, 2011 (Trial 1), then for 5 different birds starting on April 2, 2011 (Trial 2). Between these two trials, a substantial and enduring spring warming occurred, such that the thermal profile for the second trial was dramatically different from the first. To investigate the occurrence of intra-individual variation in response to different temperature regimes, the birds from Trial 1 were followed again for Trial 3, starting on April 22, 2011. However, due to transmitter failures, replicate data from Trial 1 and Trial 3 were only successfully obtained from one individual, Bird #130. Total numbers of birds with successful heart rate data were $n = 3$ for Trial 1, $n = 4$ for Trial 2, and $n = 3$ for Trial 3, with the latter including Bird #130.

Field Telemetry

Adult GWCS were captured in Davis, CA (**Supplementary Figure 1**) during the spring (January – March; $n = 10$) and autumn (October – November; $n = 9$) of 2012. During the spring, birds were primarily captured at sites A, C, and F while in fall most birds were captured at site B (**Supplementary Figure 1**). We captured birds between 0700 and 1100AM. Birds were banded with an aluminum USFWS band and a unique set of color bands for later visual identification. Heart rate transmitters were attached as above on the day of capture. After birds recovered from the effects of anesthesia, they were held overnight for monitoring in a flight cage within the aviary. We then released each bird early the following morning at the site of their capture. Following the trials, some transmitters were removed at recapture and some of them fell off and were retrieved from the field.

Individual birds were followed in the field as continuously as possible and as closely as necessary to maintain optimal signal strength. In open terrain, the quality of the signal degraded at a distance of ∼30 m, with significant reductions in operable distance associated with heavy shrubs or ravines. Thus, it was not always possible to collect data at a distance sufficient to avoid disturbance of the flock by researchers. In these cases, the equipment was attached to a tripod or other available structure and were checked every 1–3 h to ensure that the bird was still present and that the equipment batteries were still functional. For overnight recording, the equipment was left attached to an automotive backup battery, packed inside a rain-proof sack with the antenna clamped to a tripod or fixed structure. Each bird was followed for at least 2 full days after release. In the rain, transmitter batteries became waterlogged and ceased functioning for periods of hours to days. This failure sometimes also occurred when birds bathed under drip-irrigation devices that were present in the vineyards that birds frequented. Occasionally, a bird's signal was lost, usually after fleeing a disturbance, and could not be relocated for an extended period. Some transmitter frequencies were vulnerable to interference from unknown local sources such as intermittent static or total disruption. Finally, transmitters remained functional for variable lengths of time before failing and/or falling off.

Meteorological Data

Hourly data for statistical analyses were drawn from a weather station (38.536°N, 121.775°W) maintained by UC Davis that collects data for the California Irrigation Management Information System, Mesowest ID# CI006[1]. This station is located approximately 1 km north of our field site at an elevation of 18.3-m.

Heart Rate Signal Processing

Heart rate data were extracted from audio files as described above. Because signal quality was more variable for field data, heart rates were averaged over 1-min periods, retaining only bins for which more than 10 s of data were present after extracting data as previously described. One-minute bins were averaged over 60 min, at which point heart rate data were aligned with meteorological data. Hourly heart rate and temperature were averaged to obtain daily average heart rate (BPM). Averaging hourly bins, as opposed to 1-min bins, ensured that times of day in which the signal was noisiest (e.g., periods of active foraging) and therefore contained fewer 1-min data points (due to filtering) were not underrepresented in the average. Thus, all hourly bins were weighted equally regardless of the number of 1-min data points they contained.

Daily averages were defined as the 24-h period before and after sunrise. 24-h periods with <16 h worth of binned data were excluded from analysis. Because temperature was aligned with heart rate prior to averaging, only temperature data for periods with valid heart rate data were included in averages when evaluating relationships between these two variables.

Manipulation of Foraging Effort

Gambel's white-crowned sparrows were acclimated to captivity as described above, then transferred indoors to individual cages (35 cm × 40 cm × 45 cm) and kept on a continuous short-day light cycle (8L:16D) at 21–23°C. Short day lengths were chosen so all birds, whether housed indoors or exposed to natural day length were on similar light/dark schedules. Mazuri food was distributed *ab libitum* and was split evenly between two circular trays on the floor of the cage (6-in × 1-in plastic pot saucers) that could accommodate a variety of substrates in addition to food (**Supplementary Figure 3**). Grit and water cups as well as a water bottle were installed in the cage walls; wild bird seed was removed from their diet. Each bird was housed in its home cage for all training and trials but were moved into a separate, adjacent room (*hereafter*, the trial room) for training and trials. In the trial room, all cages were arranged on two

[1]http://mesowest.utah.edu

shelves, each holding two cages, which were thoroughly and evenly lit by fluorescent light fixtures positioned on the wall and ceiling. An array of 4 closed circuit television (CCTV) cameras was positioned to observe and record each bird's behavior. The room was illuminated during dark hours by a single low-intensity LED nightlight (Limelight Nightlight, Austin Innovations, TX, United States). Cameras were also equipped with infrared LEDs that gave off some visible red light.

Trial Substrate and Training

We used natural white aquarium sand (Petco, CA, United States) for our testing medium because it was difficult but not impossible for birds to displace, and the food could be easily extracted from it at the end of the experiment to determine how much had been consumed. In this way we tested for the energetic cost of self-maintenance by increasing foraging effort. Birds accessed buried food with increasing amounts of aquarium sand in their food trays.

To train birds, we initially provided individuals with trays where food was fully visible. No sand was present initially during training, but several stones of natural white aquarium gravel were used to stabilize the tray and prevent spillage. The food was spread evenly over the bottom of the tray. Sand was then added daily in small, incremental amounts to the trays with a few pieces of food remaining visible to provide birds a visual cue. Trays were changed daily at which point remaining food was weighed to verify that all birds were eating. We also recorded videos to confirm that birds were successfully finding their food and not simply displacing it from the tray. At the end of the training, all birds had learned to search for fully buried food through a tray of sand that was approximately 2 cm deep. Following completion of the training, birds were returned to the general housing room and continued to receive their food in the same trays mixed with small amounts of sand and gravel for the duration of the study.

Manipulation Experiments

Birds were moved in pairs to the trial room, so that each bird was on the same shelf with a familiar neighbor. GWCS form flocks in winter and maintaining neighbor pairs may minimize social stress (Apfelbeck and Raess, 2008, but see Banerjee and Adkins-Regan, 2011). Birds were weighed and measured as above. Individuals were allowed to acclimate to the trial room for 24 h before starting a feeding treatment. We provided 6 g of food per day divided equally between two food trays and placed in the trays at the bottom of each cage. This amount was slightly more than the maximum that any bird was observed to eat each day when consumption was measured during pilot trials. Food was delivered just before lights off each day, so for Day 1 food was placed in the day prior. We then conducted one of two randomly assigned feeding treatments ("Easy" or "Hard" **Supplementary Figure 4**) on each set of neighbor pairs by shelf. This approach allowed birds to only see other individuals from the same treatment regimes. The Easy treatment consisted of trays with food, a few pieces of larger aquarium gravel (for weight) and no sand. The Hard treatment consisted of food that was entirely buried under ∼2 cm of sand. Exposure to initial treatments was continued for 3 days. On the third and last day, birds were removed from their cages shortly after the lights came on and a blood sample was taken (as described above) within 3 min of researchers entering the room. At the end of that same day, treatments were switched so that Easy treatments became Hard and Hard treatments became Easy, **Supplementary Figure 4**, which began the second 3-day treatment exposure. Blood samples were taken for measurement of baseline corticosterone at 9AM at the start of each experiment and every 3 days throughout each trial.

Activity Scoring

We recorded bird activity continuously using CCTV throughout each experiment. Videos were automatically saved as a series of 15-min files and we used this time period as the sampling unit for scoring. We initially performed behavioral scoring using only the daytime videos from Day 2 of each treatment, which was the day prior to blood sampling. We later increased the video scoring period to include some nocturnal observations (described below). Assistants were trained to score each file. To improve consistency, we used quantitative scores based on presence or absence of a criterion for a minimum of 10 s within a 15-min video segment. Presence of foraging behavior was defined by the bird occupying a food dish for longer than 10 s (actual digging or eating could not be clearly distinguished from the low-resolution CCTV video footage). We classified the presence of a specific type of hopping behavior (i.e., hopping from perch or floor up to walls or ceiling of the cage for longer than 10 s in each 15-min video) as "escape-type" behavior because individuals did not appear to be engaged in any significant foraging, exploration, or interaction during the periods when this behavior was manifested. A similar interpretation is noted for this species by Astheimer et al. (1992), however, this "escape" behavior may also represent a stereotyped response by individuals to cope with captivity (Rose et al., 2017).

Based on prior experience and other studies of this species, we initially assumed that birds were active only during daytime, and thus, only scored daytime videos. However, night videos were later examined to verify inactivity but revealed that birds were, in fact, variably active at night. Nocturnally active birds engaged in vigorous, escape-type hopping to walls or ceiling similar to that observed during the day. Consequently, nighttime activity was estimated by sampling one 15-min video per hour throughout the 16 h dark period, beginning approximately 15 min after lights-off. The total number of samples during which escape-type hopping activity was observed (with the maximum being the total number of hours of darkness, 16) is reported as the night activity score. Due to the differences in day and nighttime sampling procedures and duration, both day and night hopping data were converted to percentages of active intervals out of the total observed during that period. Raw scores (# intervals with hopping) were used for all statistical tests, but percentages are used in figures where day and nighttime trends are compared.

Statistical Analyses

JMP Pro (v. 11.2.1) or SAS (v. 9.4) (SAS Institute, NC, United States) were used for all statistical analyses. We used

multiple linear regression to determine relationships among our variables of interest. Prior to all analyses, all variables were checked for normality and heteroskedasticity using the Shapiro-Wilks test. We further checked model assumptions by plotting residuals against predicted values.

Model Selection of Captive and Field Datasets for Heart Rate

To determine the overall linear relationship between f_H and $\dot{V}O_2$, we used multiple linear regression. We used ANCOVA to test for differences in slopes between individual birds. Data were compared using mixed effect models with a Kenward-Rogers correction. Each hypothesis was tested separately for each dataset. Allometric effects were investigated through the inclusion of body mass or tarsus length as a fixed effect and as part of an interaction variable; variables were removed using stepwise selection when they did not explain significant variance ($\alpha = 0.05$). Akaike's information criterion (AICc) was compared during model selection to ensure that the final model provided the best fit. Competing models < 2 ΔAICc are included for comparison.

Assumption 1: We compared average daily heart rate to average daily temperature controlling for Bird ID, which was nested within trial (for captive data) or season (for field data) as a random effect.

Assumption 2: Recall that this assumption was only directly testable using the captive dataset. We compared baseline corticosterone (dependent variable) to average heart rate for the 24-h period prior to blood sampling and controlled for Bird ID nested within trial as a random effect.

Based on identified relationships between heart rate and temperature, Assumption 2 was indirectly tested using both captive and field datasets. To do this, we compared baseline corticosterone to average temperature (for the 24-h period before blood sampling) with Bird ID nested within trial as a random effect for captive data. In the field dataset, it was not necessary to include Bird ID in the model testing Assumption 2 because corticosterone was only collected once.

Data Analysis of Manipulation of Allostatic Load

We used linear mixed effect models to assess the influence of treatment (Easy or Hard), treatment exposure (1st or 2nd treatment within a trial), and their interaction, including post-treatment mass change (g), baseline corticosterone (ng/mL), and qualitative activity scores. We included individual bird ID as a random effect to control for repeated sampling.

While foraging workload was the key independent variable in our manipulation experiment on allostatic load, we chose to extend our analysis to include relevant metrics like activity score and change in body mass, evaluating relationships among outcomes. In these cases, baseline corticosterone was examined as the dependent variable, while activity, change in body mass over each trial, and their interaction were included as fixed effects in a linear mixed effects model. Because day and night activity are expected to be related, separate models were run to evaluate the effect of each with relation to the other variables.

RESULTS

Heart Rate Validation

Heart rate and oxygen consumption were strongly correlated, with an overall equation of $\dot{V}O_2 = 0.07\ (\pm 0.16) + 0.0039\ (\pm 0.0002)^* f_H$ ($r^2 = 0.68$) for 6 birds (**Figure 1**). Neither log-transformation nor mass-adjustment of any kind improved this relationship. Slopes for individual birds were significantly different ($p < 0.001$) and ranged from 0.003 to 0.007. In the case of these two individuals, the data were either limited in range (consistently high heart rate) or diverged from linearity, reducing confidence in the calculation of slope. Sample sizes were insufficient to test for differences by sex and individual. However, when only sex was included in the model, it was not statistically significant.

In addition to inter-individual variation, anecdotal measurements made after the initial validation period indicated there was intra-individual variability. However, this variation was most apparent with regard to the intercept because slopes were largely similar. Pearson correlation coefficients within trials were almost universally high, and statistically redundant ($r > 0.7$). The relationship between f_H and $\dot{V}O_2$ described previously for birds vary widely (**Figure 2**). Note that the relationship for GWCS lies within the major cluster of other species.

Testing Assumption 1: Did Temperature or Manipulation of Feeding Effort Influence Allostatic Load?

Temperature significantly predicted 24-h average heart rate for captive birds ($F_{1,40.6} = 12.8$, $p < 0.001$; **Figure 3** and **Supplementary Table 1**). Morphometric variation (body mass or tarsus length) did not significantly explain heart rate within our models (**Supplementary Table 1**). Removing Bird #130 (an influential outlier that was also the only bird we were able to successfully collect heart rate from two trials) had no effect on this relationship. Ambient temperature significantly predicted daily average heart rate for free-living birds ($F_{1,69.9} = 14.0$, $p < 0.001$; **Figure 4** and **Supplementary Table 2**). Neither body mass nor tarsus length significantly contributed to the model, alone or by interaction (**Supplementary Table 2**).

Testing Assumption 2: Did Allostatic Load Predict Baseline Corticosterone?

When we compared plasma corticosterone to heart rate, tarsus, body mass, and their interaction, we found that there was no effect of average daily heart rate on corticosterone levels ($F_{1,10.1} = 0.0$, $p = 0.887$; **Figure 5** and **Supplementary Table 3**). There was also no effect of average daily daytime temperature on corticosterone levels among captive birds ($F_{1,10.9} = 0.0$, $p = 0.99$; **Figure 5A**) or wild birds ($F_{1,16} = 0.3$, $p = 0.62$; **Figure 5B** and **Supplementary Tables 4**, **5**). Corticosterone levels were not significantly correlated with average temperature for the day on which the sample was taken, or with the average temperature from the night prior to sampling (**Figure 6**).

In manipulation experiments, workload treatment did not predict baseline corticosterone ($F_{1,13} = 0.1$, $p = 0.784$). Exposure

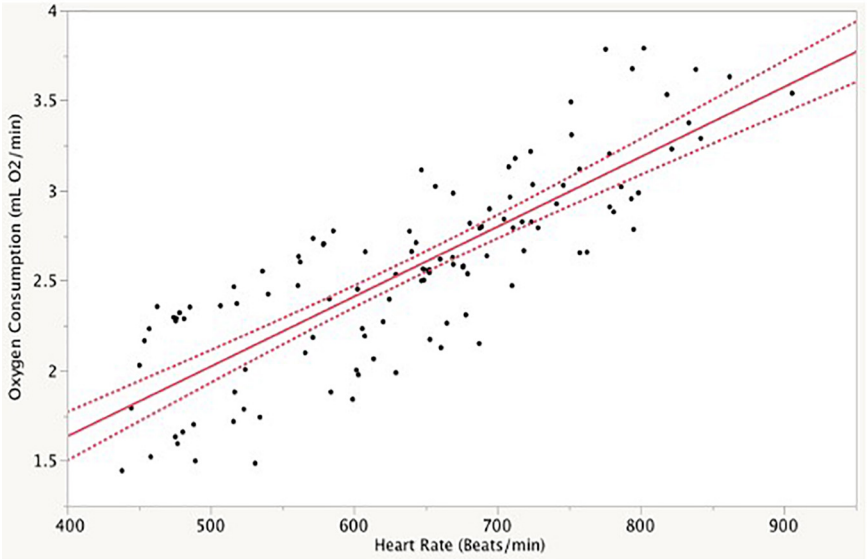

FIGURE 1 | Relationship between oxygen consumption (mL O_2/min) and heart rate (beats/min) of 6 white-crowned sparrows measured over a 2-h time period. Line indicates best fit regression with 95% confidence intervals. Each data point reflects a 5-min average.

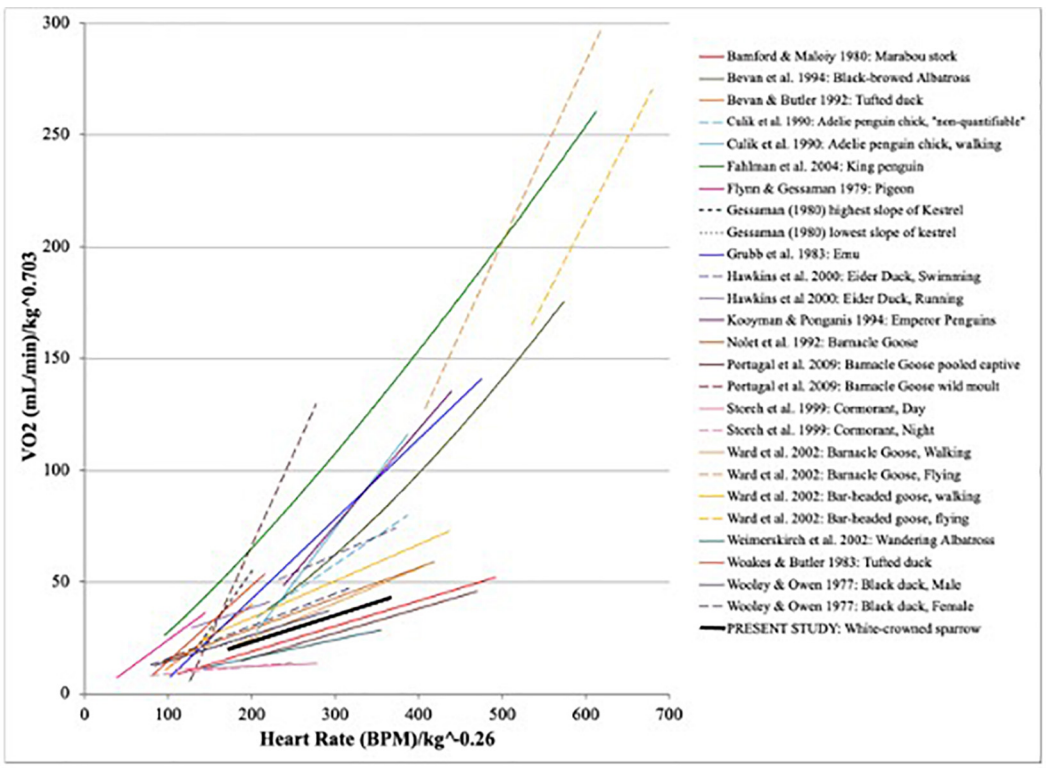

FIGURE 2 | Previously reported relationships between heart rate and oxygen consumption for birds, with the relationship derived herein overlaid for comparison. Species described are: *Leptoptilos crumeniferus* (Bamford and Maloiy, 1980), *Diomedea melanophrys* (Bevan et al., 1994), *Aythya Fuligula* (Bevan and Butler, 1992), *Pygoscelis adeliae* (Culik et al., 1990), *Aptenodytes patagonicus* (Fahlman et al., 2004), *Columba livia* (Flynn and Gessaman, 1979), *Falco sparverius* (Gessaman, 1980), *Dromiceius novaehollandiae* (Grubb et al., 1983), *Somateria mollissima* (Hawkins et al., 2000), *Aptenodytes forsteri* (Kooyman and Ponganis, 1994), *Branta leucopsis* (Nolet et al., 1992), *Branta leucopsis* (Portugal et al., 2009), *Phalacrocorax carbo* (Storch et al., 1999), *Branta leucopsis* and *Anser indicus* (Ward et al., 2002), *Diomedea exulans* (Weimerskirch et al., 2002), *Aythya fuligula* (Woakes and Butler, 1983), and *Anas rubripes* (Wooley and Owen, 1977).

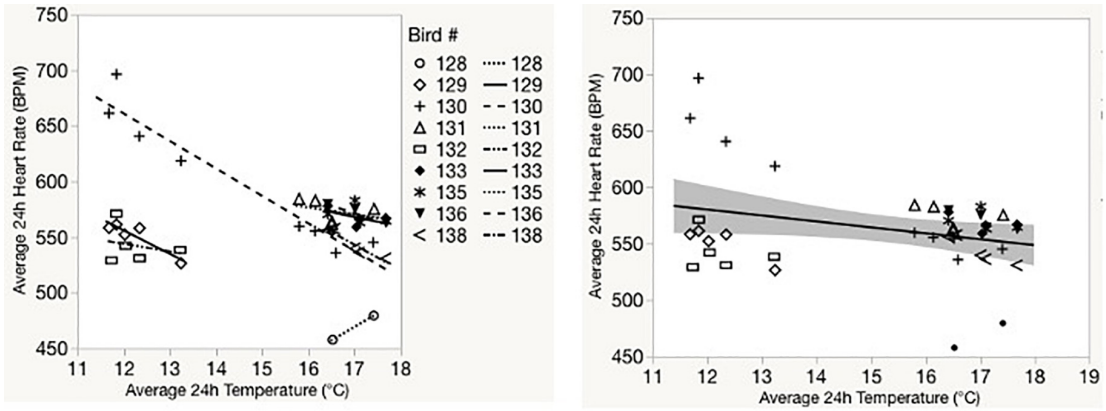

FIGURE 3 | Standard regression lines showing relationships between 24-h average heart rate and temperature for individual birds **(left)** and for all birds **(right)** in captive experiments.

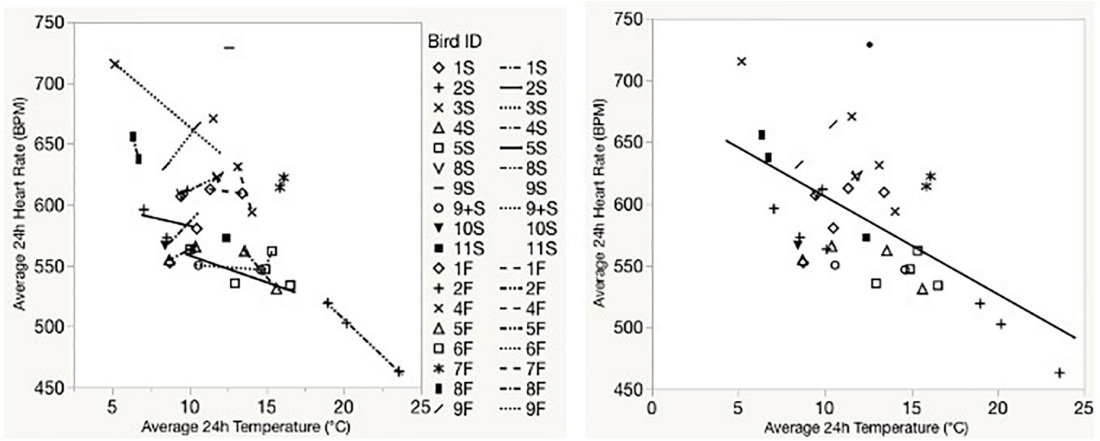

FIGURE 4 | Standard regression lines showing relationships between 24-h average heart rate and temperature for individual birds **(left)** and for all birds **(right)** in field telemetry experiments. "F" and "S" in bird ID numbers reflect data collected during fall and spring field seasons, respectively.

(1st vs. 2nd treatment) was a significant predictor in this model, with many birds having elevated baseline corticosterone during the first exposure regardless of workload treatment ($F_{1,13} = 4.9$, $p = 0.045$; **Figure 7**). The interaction between treatment and exposure was not significant ($F_{1,13} = 1.8$, $p = 0.202$).

Change in body mass differed by treatment ($F_{1,13} = 6.5$, $p = 0.024$, lsmeans difference: Easy vs. Hard est. 0.96 ± 0.38 SE; **Figure 7B**) and exposure ($F_{1,13} = 14.8$, $p = 0.002$, lsmeans difference: 1 vs. 2 est. $= -1.45 \pm 0.38$ SE; 7C), but not their interaction ($F_{1,13} = 0.1$, $p = 0.835$). Body mass declined almost uniformly during the first trial, but the Hard treatment generally incurred a larger weight loss regardless of exposure. When the Easy treatment was delivered during the second exposure, birds appeared to recover mass (**Figure 7**). The amount of food that birds ate (on D2) also differed by treatment ($F_{1,13} = 6.9$, $p = 0.021$), but not by exposure ($F_{1,13} = 3.2$, $p = 0.097$) nor by treatment*exposure ($F_{1,13} = 2.2$, $p = 0.160$). Birds ate significantly more food during the Easy regime, but the magnitude of difference was small on the first exposure and most obvious during the second. Neither workload treatment or 1st/2nd exposure was associated with significant differences in escape-type hopping during daytime hours (treatment $F_{1,13} = 1.7$, $p = 0.213$), lsmeans difference: Easy vs. Hard $= -1.39 \pm 0.11$; exposure $F_{1,13} = 2.3$, $p = 0.155$, lsmeans difference: 1 vs. 2 estimate $= -1.61 \pm 1.06$; or their interaction ($F_{1,13} = 0.1$, $p = 0.820$; **Figure 8A**) though there was a suggestive difference with potential reduction in escape-type hopping at night during the Hard treatment (treatment $F_{1,13} = 4.1$, p = 0.063; exposure $F_{1,13} = 0.0$, $p = 0.993$ lsmeans estimate: treatment: 0.87 ± 0.43 SE; exposure: 0.00 ± 0.43; **Figure 8B**).

Additional Observations From Manipulation of Foraging Effort: Relationships Among Outcomes

Corticosterone was predicted by weight change ($F_{1,12} = 9.7$, $p = 0.009$), but not escape-type hopping activity during the day ($F_{1,12} = 3.7$, p = 0.080) or at night. The interaction

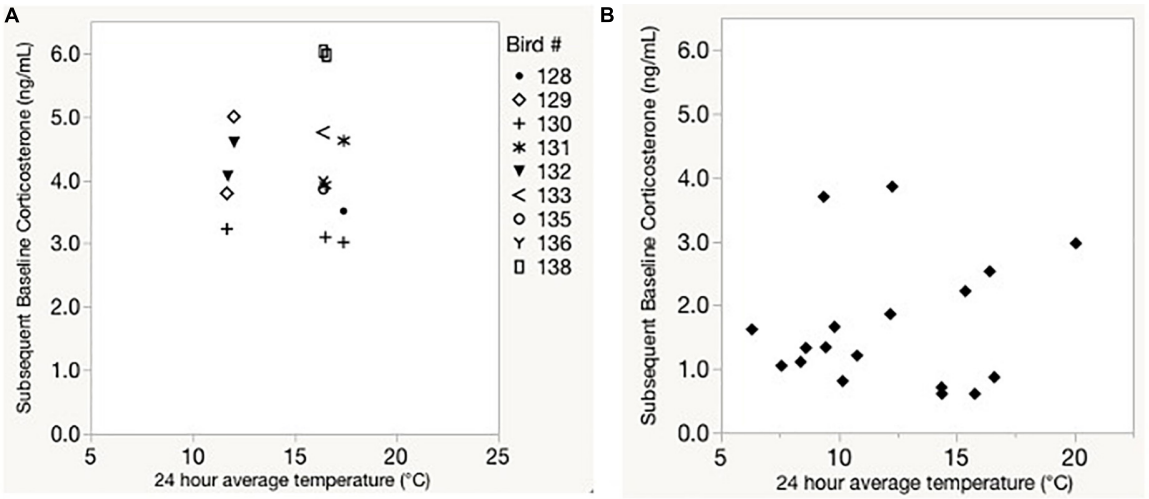

FIGURE 5 | Baseline corticosterone vs. 24 h average temperature prior to blood sampling in captive (**A**, with repeated samples identified by bird ID) and field (**B**, with each point reflecting a unique bird ID). Aviary temperature was measured *via* thermistors placed on the cage, while field temperature was recorded by a local weather station.

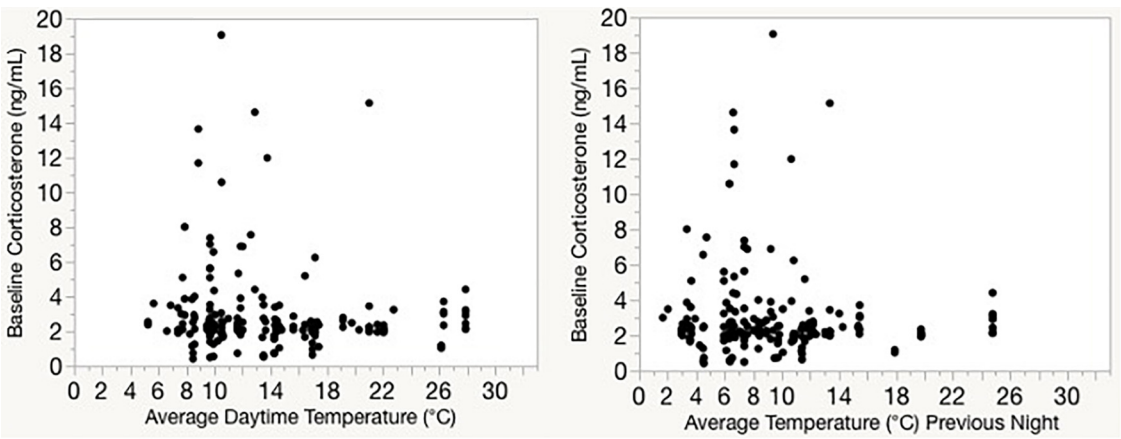

FIGURE 6 | Baseline corticosterone samples from wintering Gambel's white-crowned sparrows collected at multiple sites in Davis, CA between 2007 and 2014. Corticosterone levels are not significantly correlated with average temperature for the day on which the sample was taken **(left)**, or with the average temperature from the night prior to sampling **(right)**. Temperatures are obtained from a local weather station.

between daytime activity and weight change was also a significant predictor of baseline corticosterone ($F_{1,12}$ = 11.8; p = 0.005). The significant relationship between corticosterone and weight change was driven by the interaction term; thus, we will focus on the interpretation of the interaction. Exploration of this interaction suggests a relationship between corticosterone and activity levels that flips based on extremity of weight change. If we bin weight change into two categories, for birds that lost a lot of weight (>1.5 g), elevated baseline corticosterone was mainly associated with *high* activity levels, while among birds with more stable body mass (<1.5 g change) those with elevated baseline corticosterone were characterized by *low* activity levels (**Figure 9**). In contrast, nocturnal activity and weight change were not significantly related in any models. Furthermore, examination of the data reveals a striking negative trend between day and nighttime activity with regard to the interaction discovered with the daytime data (**Figures 8**, **9**).

DISCUSSION

Results from our validation experiments supported Assumption 1 in that changes in activity were reflected in changing $\dot{V}O_2$ and with associated change in heart rate. Heart rate telemetry further showed that variation in daily temperature influenced average daily heart rate and, thereby, allostatic load. These findings do not exclude the possibility that compensatory mechanisms, such as decreased voluntary activity (González-Gómez et al., 2011) or facultative hypothermia

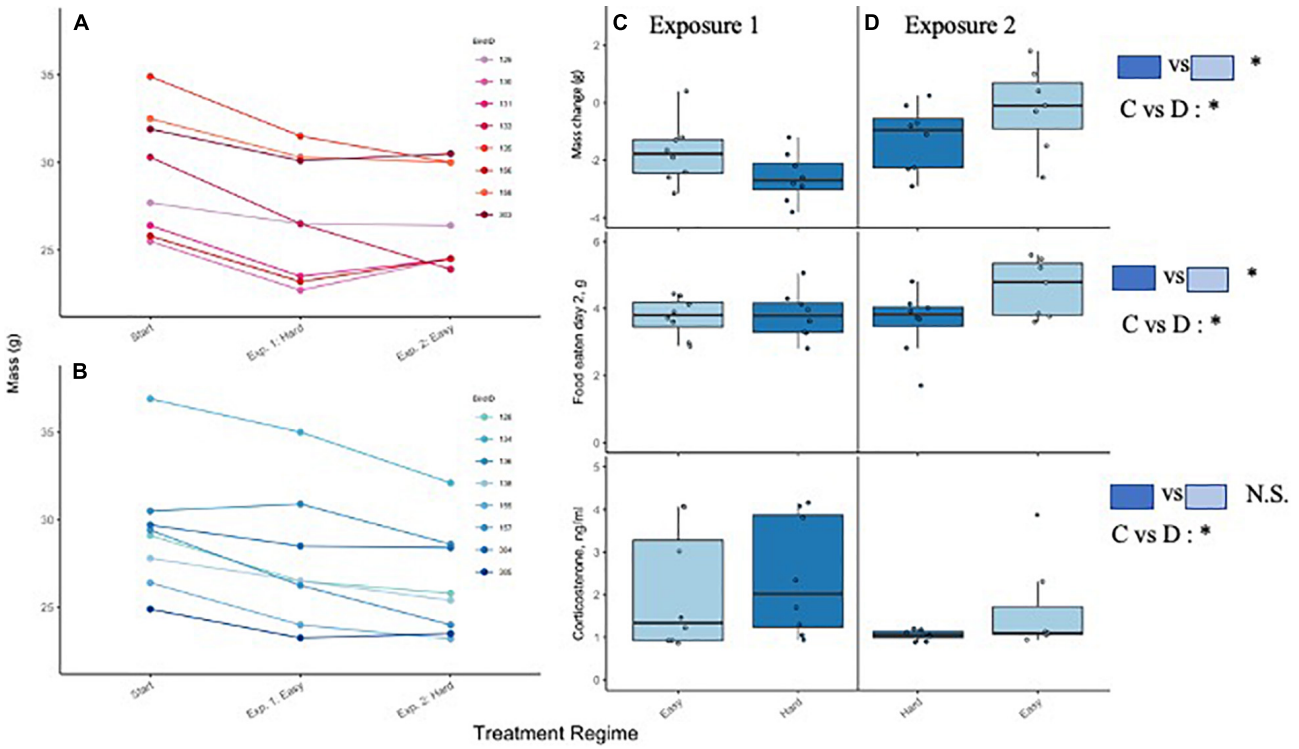

FIGURE 7 | Left hand panels. Pattern of body mass change for each bird throughout the study. Panel **(A)** shows birds that received the easy workload regime during the first exposure, followed by the hard regime; panel **(B)** shows birds that received the reverse treatment order. Right hand panels. Change in body mass during trial was affected by workload treatment and 1st vs. 2nd exposure to treatment. Change in body mass during trial was affected by workload treatment regime (**C,D** top) and treatment order (**C,D** middle and bottom). Shading reflects treatment number, with treatments separated into **(C,D)**. Lower right hand panel. Baseline corticosterone (ng/mL) was not significantly influenced by workload (feeding regime) but was elevated during the first exposure regardless of regime. Most birds lost weight under all conditions.

(McKechnie and Lovegrove, 2011), might mitigate such influence especially in relation to individual variation.

Interpretation of outcomes for manipulation of feeding effort with respect to Assumption 1 is less clear because we have no direct measure of energy expenditure. There is evidence to suggest that some birds may have engaged in compensatory strategies to reduce impact of the hard treatment on allostatic load, e.g., by eating less food or by reducing activity at night. However, these trends were far from clear or dramatic, with a much more sizeable influence of 1st/2nd exposure and associated interactions. The fact that most birds lost weight during most treatments, suggests that something other than behavioral compensation for increased workload is at play. However, we cannot eliminate the possibility that some compensation may have mitigated the impact of workload treatment on allostatic load.

Assumption 2 was not supported by our data in any experiment. We found no association between baseline corticosterone and allostatic load using either direct measurement of heart rate (aviary heart rate telemetry) or by inferring influence of temperature on allostatic load (aviary and field telemetry along with other field data) based on support for Assumption 1. We also found that increased foraging workload did not significantly affect baseline plasma corticosterone levels in captive birds. Body mass and tarsus length explained little of the variation in our models and thus were not considered influential either as determinants or as mediators/moderators of the response variables used to test this assumption. Nonetheless, body size is a potentially important source of error. Where inter-individual variation in heart rate is influenced by heart size, for example, rather than factors related to allostatic load, this variation impairs the power of the model we used to compare data between individuals in tests of Assumption 2. The influence of temperature on allostatic load may also vary with body size, but likely less systematically due to other factors such as microhabitat or feather condition. While this source of error is important, our failure to detect a relationship between baseline corticosterone and allostatic load in multiple different tests of Assumption 2 provides more robust grounds for its rejection.

Potential for Relationships at Other Levels of the Hypothalamic-Pituitary-Adrenal Axis

The framework underlying the allostasis model specifically suggests that circulating glucocorticoids should track allostatic load. However, in outlining the model,

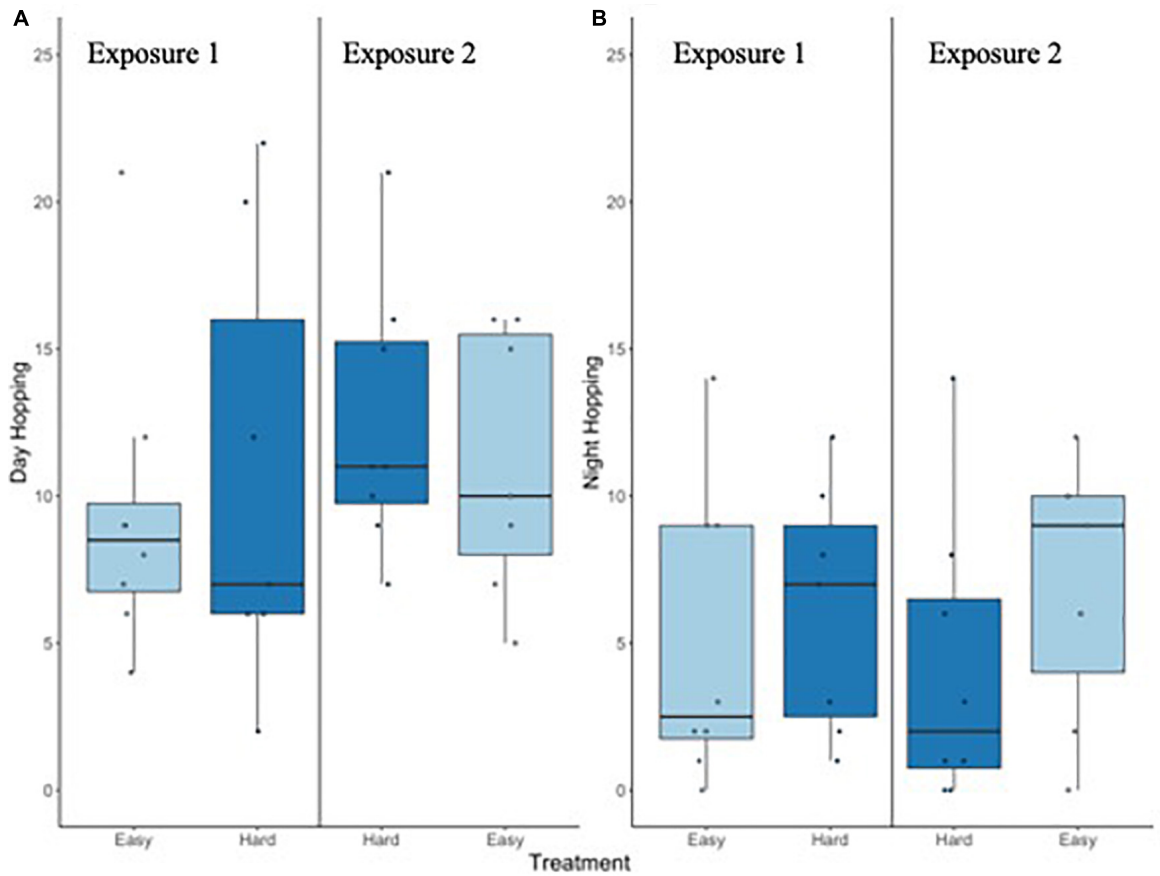

FIGURE 8 | Number of 15-min intervals in which escape-type hopping was observed during daylight hours (**A**; all intervals scored) and at night (**B**; sampled hourly) on the day prior to blood sampling. This type of activity was not significantly affected by workload regime or exposure.

McEwen and Wingfield (2003) also stated that glucocorticoids function within a suite of "interconnected hormonal mediators." Changes in glucocorticoid transport, local enzymatic control, receptor density or responsiveness may act independently to effect change on the organism (see also Ball and Balthazart, 2008). The primary plasma transport protein for glucocorticoids, corticosteroid binding globulin (CBG), is believed to both facilitate transport and prevent its action (Breuner and Orchinik, 2002), with the latter being the effect most widely accepted as outlined in the free hormone hypothesis (Mendel, 1989). Local control of hormone concentrations can occur through enzymatic breakdown or reconstitution *via* expression of 11β-hydroxysteroid dehydrogenase enzymes (Tomlinson et al., 2014; Pérez et al., 2020). Other molecules also co-vary with circulating levels of corticosterone across seasons (Breuner and Orchinik, 2001), but some changes can occur even when total corticosterone remains constant (Lynn et al., 2003; Sesti-Costa et al., 2012; Krause et al., 2015). Hence, the possibility that Assumption 2 may be fulfilled at a molecular level not explored in this study cannot be eliminated, and further investigation in this area could be a fruitful direction for future investigation. Nonetheless, systemic effects of hormone action are most parsimoniously regulated by change in hormone level. Tissue-level changes in hormone action are far more likely to be relevant to fine-tuning local control.

Context on Validation of Heart Rate as a Measure of Energy Expenditure

The oxygen consumption values observed for Gambel's white-crowned sparrows are within the range expected for this species. King (1964) reported a standard metabolic rate for *Z. l. gambelii* of 2.43 mL/g*hr (1.16 mL/min) based on the average mass reported for his birds. This value is expectedly lower than the minimum reported here (mean minimum = 1.68 ± 0.13 mL/min) for fed birds at rest. Most studies comparing f_H to $\dot{V}O_2$ tend to vary widely (**Figures 1**, **2**) and did not typically compare slopes between individual animals. When individual slopes were tested, the application of a combined equation was sometimes rejected when individuals differed significantly. However, heart rate is not expected to explain 100% of the variance in oxygen consumption, *a priori*. While many studies have found consistent slopes among individuals, others found broad differences (Gessaman, 1980), or found slopes differ with flight (Ward et al., 2002) or captivity (Portugal et al., 2009). Even with the considerable variance in energetic expenditure that cannot be captured using heart

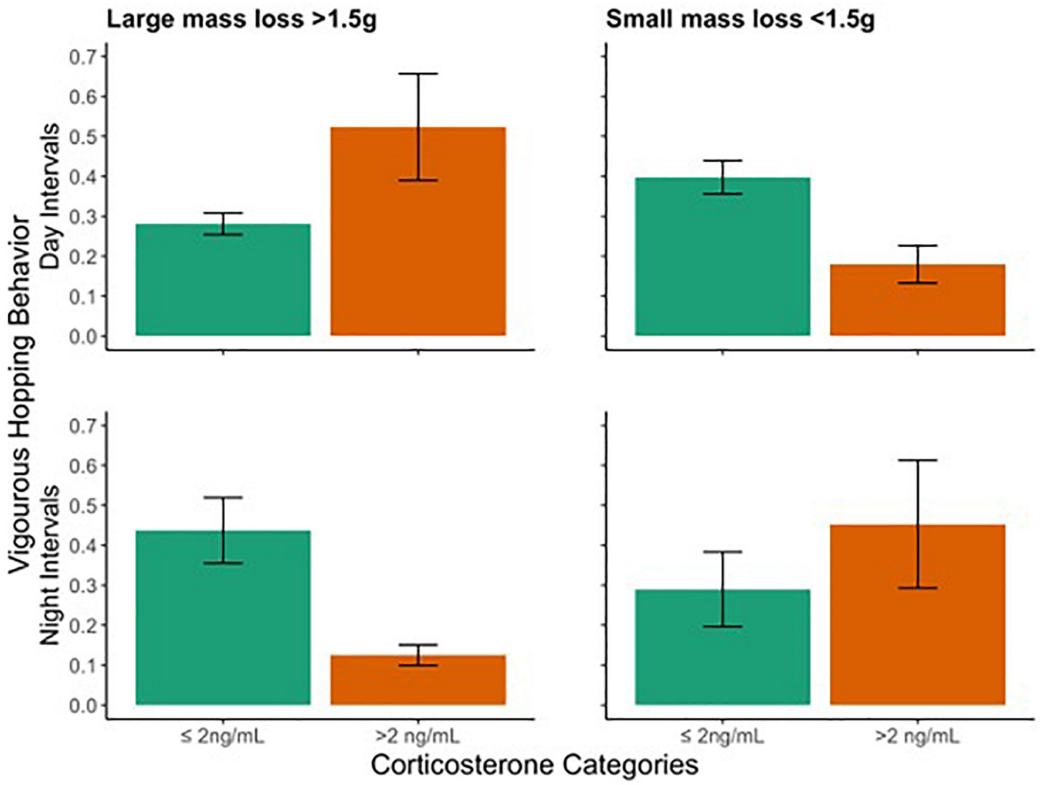

FIGURE 9 | Difference between day (top panels) and night (bottom panels) escape-type hopping behavior with regard to baseline corticosterone for binned categories of large **(left)** and small **(right)** mass loss. Bars show means ± SEM for each group. Activity data are displayed as a percent of intervals counted. While no significant effects or interactions relate nighttime activity to baseline corticosterone, overall trends are opposite to the significant interaction between daytime activity, weight change, and corticosterone.

rate there is still a positive relationship between f_H and $\dot{V}O_2$ supported by the data from our study organism. Furthermore, if we adjust for large differences in body size across studies using mass exponents of 0.703 for avian field metabolic rate (Tieleman and Williams, 2000) and −0.26 for heart rate (White and Kearney, 2014), the observed relationship we found is consistent with other studies of different species, particularly when (mass-adjusted) ranges of heart rates were examined. While it is not possible to draw specific conclusions from comparisons across such a wide range of phyla, based on this rough visual comparison our data bear the greatest similarity to relationships established for walking ducks and geese (**Figure 2**).

Because $\dot{V}O_2$ is itself a proxy for direct calorimetry, even when (or if) heart rate varies predictably with $\dot{V}O_2$, the prediction of energy expenditure is necessarily imperfect (Walsberg and Hoffman, 2005, 2006). Even direct calorimetry itself is far from error-free, so the source of discrepancies between direct and indirect methods may never be fully understood (Speakman, 2014). In any case, whether reported as milliliters of oxygen or kilojoules, some error in estimating true energetic expenditure is a consequence of indirect calorimetry.

To our knowledge, no study has investigated a direct relationship between heart rate and energetic expenditure *via* direct calorimetry. Heart rate, when used as a measure of energy expenditure, is inarguably a blunt instrument. Small changes in energy expenditure, or small differences between animals may well be undetectable by f_H. However, to the extent that relationships between environmental factors and f_H are observable, the data presented in this paper justify the inference of a corresponding relationship between the same environmental factors and energy expenditure overall. This finding is in accordance with other studies that have utilized heart rate telemetry to evaluate energy expenditure in songbirds (Bowlin and Wikelski, 2008; Cyr et al., 2008; Steiger et al., 2009; Bisson et al., 2011, 2009; Romero and Wingfield, 2016). While heart rate data are not converted to units of oxygen consumption using the equation validated here, the consistency of correlation, particularly of the slope, both supports Assumption 1 and justifies the use of heart rate as a proxy measurement for energy expenditure when testing the model of allostatic load.

Context on the Response of Glucocorticoids to Weather Changes

Several studies have looked for relationships between glucocorticoid hormones and changing temperatures with variable results (reviewed in de Bruijn and Romero, 2018). Among relevant studies (excluding heat stress, heterotherms, and early development), the impact of temperature on circulating corticosterone remains mixed with, some finding glucocorticoids do vary with temperature (Huber et al., 2003; Frigerio, 2004;

Dorn et al., 2014) while others did not or found responses varied with other factors (Wingfield et al., 1996, 1997, 2003; Romero et al., 2000; Lobato et al., 2008; Knutie and Pereyra, 2012). In contrast with the present study, all unequivocally positive results used fecal corticosteroid metabolites to evaluate glucocorticoid response, which may be an important distinction. While stable changes in baseline corticosteroid levels are expected to be observable in fecal metabolite levels, many other kinds of change, e.g., altered responsiveness to minor stressors in the environment or the amplitude of circadian peaks, can influence fecal metabolite levels but go undetected in an instantaneous sample of blood plasma (Ninnes et al., 2010; Sheriff et al., 2010). Fecal samples contain an amount of corticosteroid metabolites accumulated over the time period during which food material has passed through the gut of the animal. Thus, all endocrine events that occurred during this time can influence the amount of metabolites in a sample. Additionally, any factor that affects gut transit time can also influence the amount of metabolites found in any given sample (Goymann et al., 2006). If glucocorticoids were to increase abruptly in response to reduced perturbation resistance potential, as in the alternative to Assumption 2, this increase could be evident in fecal metabolite sampling but not in instantaneous plasma measurements of hormone, depending on sample timing.

Much of the other evidence commonly cited in favor of a role for glucocorticoids in mediating responses to weather is drawn from studies of extreme weather events (Rohwer and Wingfield, 1981; Wingfield et al., 1983; Schwabl et al., 1985; Rogers et al., 1993; Smith et al., 1994; Raouf et al., 2006). In these situations, cold temperature is only one part of a larger environmental perturbation that may involve compromised shelter and/or restricted access to food. Food restriction, in particular, is often cited as the most likely explanation for the observed hormone response (Schwabl et al., 1985). Additionally, changes to corticosterone were observed in the absence of notable temperature change (Rogers et al., 1993; Raouf et al., 2006) or appeared to be dependent on season or breeding condition (Wingfield et al., 1983; Ramenofsky et al., 1992). Other studies on white-crowned sparrows found limited effects of cold temperature in males of one subspecies (Wingfield et al., 1997), but no differences in corticosterone in any other group. Because their tests were run concurrently with photostimulation, it is interesting to note that temperature itself had direct inhibitory effects on gonadal recrudescence, which should minimize allostatic load by reducing the cost of gonadal growth. This potential compensatory strategy merits further consideration because it suggests that other physiological processes might, similarly, respond to temperature in these birds.

Even though a relationship between energy expenditure and temperature was observed in this study, if such direct physiological compensation were to differ among individuals, this could detract from the robustness of energy expenditure as an estimate of allostatic load. The presence of some confirmed relationships among negative results (Wingfield et al., 1997; Romero et al., 2000) suggests that there may be a threshold, or multiple interacting factors influencing how circulating glucocorticoids interact with temperature. A recent study of greylag geese (*Anser anser*) showed sex-based variation in the response of heart rate and core body temperature to ambient changes (Wascher et al., 2018), which supports the potential for complex interactions to mask impacts of temperature on corticosterone *via* changes in allostatic load.

Context and Additional Observations on Responses to Workload Manipulation

When we manipulated foraging workload, we observed no change in baseline plasma corticosterone. This contrasts with a recent test of workload on feather corticosterone [in growing feathers of female ducklings, *Anas platyrhynchos* (Johns et al., 2018)]. These authors found that when elevation of allostatic load was induced by obstacles and adding weight to individuals, corticosterone levels of feathers growing throughout the treatment period were increased. Like fecal corticosterone, feather measurements represent a longer term "sum" of corticosterone change in comparison with the "snapshot" of a plasma sample. In addition, the manipulations in this study were more extreme, likely inducing allostatic overload. The lack of associations of allostatic load with baseline plasma corticosterone observed in this study are, therefore, not inconsistent with existing data. Curiously, we did find a positive effect on baseline corticosterone in this study – not of workload treatment but of the first exposure, regardless of treatment. We also observed an interaction with the concurrent outcomes of daytime escape-type activity levels and weight loss when these were treated as independent variables that could influence corticosterone levels.

The effect of first exposure regardless of treatment suggests that something about the birds' introduction to the experimental room had an effect on baseline corticosterone that persisted through the first 3 days of treatment. Even though plasma samples after the first exposure were higher than those sampled later, the direction of effect cannot be inferred because pre-trial samples were not collected. Initial exposure could have increased baseline corticosterone levels, after which they slowly returned to previous levels, but it is alternatively possible that hormone levels may have fallen steadily, on average, throughout the two trials.

Prior to experimental trials, all birds had been trained in the experimental room for periods of 1 week or more, so nothing about the environment was novel. If the training had created a negative association, we would expect birds to respond specifically to the "hard" workload regime on which they were trained at that time – this was not observed. While the effect of translocating birds within captivity has not been formally studied, the large elevation in baseline corticosterone associated with being brought into captivity has been found to extend for at least 3–4 days (Wingfield et al., 1982). If the experience of changing rooms were perceived as a "stressor" of this type, though much smaller in magnitude, then the time frame of acclimation does fit with the pattern observed here. However, given the regularity with which birds are transferred between rooms in captive experiments, it seems unlikely that a response of this kind would have eluded detection throughout the extensive history of study in this species.

Alternatively, baseline corticosterone levels may have steadily declined in response to the room transfer. The experimental room contained only 4 birds, whereas the housing room contained 16 birds in individual cages. The effect of housing density on songbirds is poorly understood but its effect on corticosterone has been found to depend on social rank (Nephew and Romero, 2003). Such responses are therefore likely to vary with social structure and seasonality. Our birds were maintained on short days, a photoperiod that is normally associated with wintering conditions when white crowned sparrows remain in loose flocks. The proximity of caged conspecifics is arguably similar to how birds are typically spaced in the field, at least during daylight hours. Average baseline plasma corticosterone levels measured in the context of other studies with housing conditions and photoperiod similar to those utilized here are comparable to the intermediate-to-higher values reported in this study (M. Ramenofsky, J.S. Krause, pers. com., e.g., Busch et al., 2008a,b). Taken together, these data show that a steady decline of corticosterone from a higher level prior to exposure 1 cannot be eliminated.

The interaction between weight loss and daytime activity level in predicting baseline corticosterone is unexpected but potentially meaningful. Astheimer et al. (1992) performed an entirely different experiment, in which the same species of birds were implanted with corticosterone, fed either *ad-libitum* or a restricted diet, and monitored for activity. These birds exhibited a relationship between corticosterone treatment and activity that was opposite for feed restricted birds compared with those fed *ad-libitum*. Specifically, corticosterone-implanted birds both increased activity during feed restriction, but were less active than controls during the *ad-libitum* feed regime. A previous study (Buttemer et al., 1991) with similar conditions showed that fasted and corticosterone-implanted birds all minimized metabolic rate by reducing episodes of nighttime arousal, but there was no additive effect of corticosterone-implantation with fasting; i.e., they either conserved at night or they did not. In the present study, neither high weight loss nor high corticosterone predicted night activity and the interaction between the two was also not statistically significant. While the study conducted by Astheimer et al. (1992) is radically different in design from the experiment performed here, there is logic in the comparison. In either interpretation of the correlation between baseline plasma corticosterone levels and order of exposure described above, the most likely explanation is one in which a classical "stressor" of some kind causes corticosterone levels to increase. Hence, although corticosterone is examined here as an outcome in reference to activity and change in body mass, it may be more appropriate to consider an alternate model in which baseline corticosterone levels, influenced by some stressor rather than implantation, may predict escape-type activity in a manner dependent on the nutritional status of the animal. When organized in this way, the outcomes of these two studies, at least with respect to daytime activity, are strikingly similar.

Conditional effects of the kind observed here are gaining recognition as a likely explanation for otherwise contradictory data, particularly with regard to the relationship between glucocorticoids and locomotion, which is also highly relevant to site abandonment following environmental perturbations, i.e., the "take it or leave it" decision (Wingfield and Ramenofsky, 1997). In mountain white-crowned sparrows (*Z. l oriantha*), corticosterone results in birds delaying return to a territory abandoned in response to bad weather conditions, but enhances foraging (rather than inducing abandonment) during fair weather (Breuner and Hahn, 2003; Breuner et al., 2013). In black-legged kittiwakes (*Rissa tridactyla*) corticosterone implants increased foraging and flying behavior only in birds with good body condition (Kitaysky et al., 2001a,b; Angelier et al., 2007). Similar data also exist for rats, in which corticosterone increases wheel-running only in food-restricted animals and furthermore is permissive of the increase associated with food restriction based on comparison with adrenalectomized controls (Duclos et al., 2009). Glucocorticoids are also permissive to locomotor effects of stimulant drugs in rodents (Marinelli et al., 1997), so all of these examples, inclusive of the present study, may reflect a permissive mechanism by which endocrine and environmental signals may be integrated to result in an optimal behavioral effect. A permissive role in mediating the effect of weight loss does not explain the additional and opposite effect of corticosterone in reducing daytime activity for animals that maintained weight (or were fed ad lib in Astheimer et al., 1992). This may occur *via* a separate but, similarly, integrative mechanism. Neuroendocrine peptides such as corticotropin releasing factor can also influence locomotor activity (Morley and Levine, 1982; Maney and Wingfield, 1998; Romero and Wingfield, 2016), and the extent to which such other factors might be involved in further mediation is unknown.

The three-way interaction observed in this study, particularly the important role played by weight loss as a conditional factor in determining the effect of corticosterone, is fundamentally compatible with a view of the model of allostatic load in which resource availability (E_G in the original model), is equal in importance to allostatic load. Organization, evolution, and conceptualization of such a complex, integrated regulatory system within the framework of allostasis needs more investigation in the context of the organism in its environment.

FUTURE DIRECTIONS

This study finds evidence to support the hypothesis that fluctuations in average daily energy expenditure, here considered as allostatic load, occur and are related to ambient temperature in wintering GWCS. Despite multiple tests, no evidence is found of any relationship between routine fluctuations in allostatic load and circulating baseline corticosterone, as predicted by Wingfield and McEwen's (2003) model. While it is possible that this negative result is spurious, or that other levels of the hypothalamic-pituitary-adrenal axis and/or associated proteins may be the true targets of regulation, these data are consistent with other studies in suggesting that a relationship, if or where present, may not be as simple or direct as portrayed in the original model.

Current results call for a re-examination of this model in view of the challenges presented here. If baseline circulating corticosterone does not increase gradually with routine allostatic load, there may instead be a threshold at which a response is generated and this threshold may vary across individuals in relation to condition, resource availability, and other factors that influence risk of energetic crisis. This raises the possibility that individual perturbation resistance potential may play a key role in determining when a threshold of allostatic load and available resources is reached. This would signal critically reduced perturbation resistance potential thus triggering a glucocorticoid response and the emergency life history stage. Further work examining the response of glucocorticoids to allostatic load should anticipate interactions that may serve to define that threshold.

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 reviewed and approved by Institutional Animal Care and Use Committee, University of California, Davis.

AUTHOR CONTRIBUTIONS

KW designed the investigations, conducted the research, and writing of the final manuscript. SA analyzed the data and produced many of the figures. JW was involved in the design of the investigations, provided funding and was involved in writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.855152/full#supplementary-material

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Suboptimal Embryonic Incubation Temperature Has Long-Term, Sex-Specific Consequences on Beak Coloration and the Behavioral Stress Response in Zebra Finches

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Secondary sex characteristics, like beak color in some avian species, have indirect impacts on reproductive success, as they are considered to be honest indicators of condition, immunocompetence, and developmental history. However, little is known about the long-term effects of environmental perturbations on the production and maintenance of these secondary sex characteristics in avian species. In zebra finches (*Taeniopygia guttata*), redder beaks indicate increased carotenoid expression and implantation into beak tissue, and female zebra finches prefer males with pronounced bright red beaks as a mate. The present study examines the long-term effects of embryonic incubation temperature on the maturation of beak color in zebra finches. We also investigated the effects of embryonic incubation temperature on sensitivity to a handling and restraint stressor in adulthood. Specifically, the aims of this study were to examine: (1) whether suboptimal incubation temperatures affect the timing of beak color development and color characteristics before and after sexual maturity, (2) if repeated handling causes short-term changes in beak color and whether color changes are related to embryonic thermal environment, and (3) how thermal stress during incubation alters future responses to a repeated handling stressor. Zebra finch eggs were randomly assigned to one of three incubators: "Control," "Low," or "Periodic Cooling." Beak color (hue, saturation, and value) was quantified before [45, 60, 75 days post-hatch (dph)] and after sexual maturity (95 dph), as well as after repeated handling stress later in adulthood (avg of 386 dph). We found that there were age- and sex- specific effects of incubation treatment on beak hue, where females from periodically cooled eggs had decreased hues (redder) in adulthood. Additionally, eggs laid later in a clutch had decreased beak saturation levels throughout life regardless of incubation environment. We found that females had lower beak hue and saturation following a capture and restraint stressor, while males showed increased beak saturation. Lastly, males subjected to the Low incubation treatment had relatively higher activity levels during restraint than those in the Control group. Overall, these findings suggest that fluctuating incubation temperatures combined with repeated, short-term stressors can have significant, sex-specific effects on sexual ornamentation and behavior.

Keywords: ornamentation, incubation temperature, beak coloration, zebra finch (*Taeniopygia guttata*), fluctuating temperature, behavioral stress response

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INTRODUCTION

Anthropogenic disturbances to the environment such as climate change, habitat destruction, and introduction of environmental toxicants and pathogens pose novel threats to an organism's health and can act as stressors [stimuli that have the potential to inflict damage at a molecular, cellular, organ, or organismal level (Wada, 2019)]. It is crucial to identify and understand the impacts of such stressors in order to relieve and conserve at-risk species (Carey, 2009). One major concern of biologists is climate change, as global annual temperature has increased, on average, at a rate of 0.08°C per decade since 1880 (NOAA, 2022). Furthermore, climate models predict that not only absolute temperature, but temperature variability will increase in the future (Bathiany et al., 2018), which may expose various species to temperature ranges that they have not previously encountered. Yet, many published laboratory studies that investigate the effects of temperature use absolute temperature changes (i.e., static increases/decreases in mean temperature), which may not give an accurate depiction of temperature fluctuations that occur in nature. One major developmental perturbation for avian species is temperature variation/fluctuations experienced during the incubation period. Parents of most avian species sit on their eggs and use their bodies to transfer heat to developing embryos (Bertin et al., 2018), ranging from temperatures of 36 to 40.5°C (Lundy, 1969; Conway, 2000), with 37–38°C being the optimal temperature range for normal development of most avian species (French, 2009). When parents leave the nest to forage, the egg is often susceptible to periodic cooling events until the parents return to continue incubating. Although it is difficult to mimic these fluctuations in the laboratory (Valenzuela et al., 2019), studies that account for fluctuating temperatures can shed light on the conditions that force parents to leave the nest more often, therefore resulting in high incubation temperature variability, which can influence development, survival, and reproductive abilities and attractiveness of the offspring.

In birds, secondary sex characteristics such as plumage, beak color, and song can strongly influence reproductive success. These ornamental traits can serve as signals to a potential mate to indicate overall quality of the contender and have also been shown to reflect an individual's developmental history (Merrill et al., 2016). For instance, male zebra finches (*Taeniopygia guttata*) who received high quality food as young had larger cheek patches and were preferred by females than ones received a control diet (Naguib and Nemitz, 2007). Although secondary sex characteristics fully develop by sexual maturity in birds, some also change in relation to an individual's condition (Rosenthal et al., 2012) and can indicate current condition of a mate. For example, unlike plumage, vascularized beaks are particularly susceptible to environmental perturbations over time (Schull et al., 2016). At a molecular level, beak color is derived from carotenoids (McGraw, 2006), which are lipid-soluble pigments synthesized by plants, algae, bacteria, and fungi. These carotenoid pigments that are solely obtained from diet, which in turn, circulate in the bloodstream and deposit into the highly vascularized beak tissue (Pérez-Rodríguez et al., 2010). Beak color in male zebra finches has been correlated with defense against parasites (Hamilton and Zuk, 1982; McGraw and Hill, 2000; Van Oort and Dawson, 2005) and resistance to oxidative damage (Pérez-Rodríguez et al., 2010). Beak color can change rapidly, as carotenoids are quickly deposited into the living tissue in the beak (Ardia et al., 2010; Rosenthal et al., 2012; Merrill et al., 2016), which makes beak color a well-suited indicator of an individual's current condition to a potential mate. Additionally, individuals that maintain their beak color in the face of a stressor are deemed resistant to that specific stressor. This aforementioned stress resistance is defined as an organism's ability to elicit anti-damage mechanisms (e.g., behavioral, physiological, or cellular responses to avoid persistent damage), so that they are more resilient to perturbations and overall performance is unaffected (Wada, 2019; Wada and Coutts, 2021). Therefore, if males can maintain a red and bright beak color despite facing a particular stressor, such as decreased food availability/quality, disturbances in environmental temperatures, and/or repeated handling or human interference, then they are considered resistant to that stressor, meaning better at regulating carotenoid distribution throughout the beak to attract a mate. Thus, male beak color can signal stress tolerance as well as developmental history, where a female can choose males with an attractive phenotype and/or genes to further pass on to offspring.

While stress resistance can be observed in beak color development and maintenance at the expense of attracting a suitable mate, stress tolerance can also be observed by the magnitude of a stress response an individual displays and its consequences. In response to a stressor, vertebrates elicit stress responses to escape or cope with that stressor, and these responses vary depending on the severity and persistence of the stressor in the environment. These include sympathetic nervous system and adrenocortical responses, prompting release of catecholamines (epinephrine and norepinephrine), and glucocorticoids (cortisol and corticosterone) into the bloodstream. Persistent physiological stress elicited during development have been shown to have long-lasting effects in avian species, including effects on body mass (Wada et al., 2015; Zito et al., 2017) and song learning (Lindström, 1999; Buchanan et al., 2003; Spencer et al., 2005; Wada and Coutts, 2021). In addition to the adrenocortical responses traditionally examined in relation to developmental stress, organisms display behavioral stress responses (e.g., anxiety-, fear-, and depression-related behaviors) due to variations in hormone signaling (Adkins-Regan, 2005; Myers et al., 2017). Although there are more studies that examined the effects of developmental nutritional stress and incubation temperature on corticosterone (CORT) levels, relatively little is known about how a developmental stressor affects behavioral stress responses later in life (Wada and Coutts, 2021) and how these short-term behavioral stress responses can impact secondary sex characteristics.

To address knowledge gaps related to stress cross-tolerance and effects of developmental conditions on secondary sex characteristic in adulthood when fitness-related traits like beak color would be most important, we explored the long-term consequences of fluctuating and low embryonic incubation temperatures on the maturation of beak color and sensitivity to a capture and restraint stressor in zebra finches. Specifically,

we assessed three aims to elucidate the relationships between developmental stress, secondary sex characteristics, and the behavioral stress response: (1) the effects of suboptimal embryonic incubation temperatures on the development of beak hue, saturation, and value from juvenile stages to sexual maturity, (2) the short-term changes of beak coloration following a repeated capture and restraint (Cockrem et al., 2008; McGraw et al., 2011) associated with a behavioral response test [a "handling bag test" adapted from Martin and Réale (2008)] on beak color maintenance, and (3) the consequences of suboptimal embryonic incubation temperatures on responses to a repeated handling stressor. While the impacts of developmental stress may appear negative, there is increasing evidence for an adaptive role of low-level developmental stress in shaping animal phenotype throughout life (Crino and Breuner, 2015; Briga et al., 2017; Krause et al., 2017). Considering a fluctuating incubation temperature as a mild developmental stressor as opposed to constantly low incubation temperature, we hypothesized that fluctuating incubation temperatures promote cross-stress resistance (i.e., further resistant to future stressors) later in life when individuals are exposed to a capture and restraint stressor, as stress in early life has been shown to prepare an individual to future stressors in adulthood (Hoffman et al., 2018). Because developmental stress has been shown to have sex-specific effects on offspring phenotype in zebra finches where females are more negatively affected (Wada et al., 2008; Zito et al., 2017), we predicted that females incubated in the consistently low and periodic cooling incubation treatments would exhibit a more red beak color (increased hues/less attractive) across time, and short-term, repeated capture and restraint would cause a temporary decrease in female beak hue. Ultimately, the correlations between a developmental stressor and the impacts on an individual's behavioral stress response are largely unknown aside from bird song (Buchanan et al., 2003; MacDougall-Shackleton and Spencer, 2012). Thus, the purpose of this study is to correlate a developmental stress, in the form of fluctuating embryonic incubation temperatures, experienced in early life to beak color maturation and behavioral stress response profiles of individuals later in life.

MATERIALS AND METHODS

Animal Models and Husbandry

The animal husbandry and thermal manipulation protocols in this study have been extensively described in Rubin et al. (2021). Briefly, fertile eggs from 23 pairs (average of 3.7 eggs per nest) of zebra finches (*Taeniopygia guttata*) housed in at Auburn University, AL, United States were collected from April 2018 to December 2018. All breeding pairs had *ad libitum* access to seed [Kaytee Supreme (Finch) Chilton, WI, United States], water, grit, and cuttlefish bone pieces. Breeding pairs were given a tablespoon (9.50 ± 0.39 g) of egg food (boiled egg, bread, and cornmeal mixture) daily. Once a week, they were provided with spinach and a probiotic supplement (Bene-Bac, PetAg) mixed into egg food. Each breeding pair was provided an external nest box (19.5 cm × 14.5 cm × 14.5 cm) and nesting materials of shredded paper and irradiated hay (Rubin et al., 2021). To stimulate breeding, the birds were spritzed with water until egg laying began, as zebra finches use rainfall as a cue to initiate breeding (Zann et al., 1995). Each nest box was checked daily between 1,000 and 1,200 h for freshly laid eggs. Once collected, eggs were labeled with a non-toxic marker to reflect nest origin and laying order, and eggs were randomly assigned to one of three incubators (Brinsea Octagon EX incubators, Brinsea Products Inc., Titusville, FL, United States): (1) an incubator with a constant temperature of 37.4°C further referred to as "Control," (2) an incubator that periodically cooled eggs by powering off five times a day for 30 min every 2 h further referred to as "Periodic," and (3) an incubator with a constant low temperature of 36.4°C, which was the average temperature of the Periodic treatment, further referred to as "Low." There was one incubator per experimental group. Each incubator was programmed to hold a constant humidity reading of 55%. Temperature readings on the incubators were confirmed using thermometers (Thermco, 25/45°C ACCI310) for the Low and Control incubators. The lowest average temperature that the Periodic incubator reached during periodic cooling events was 29°C. After hatch, hatchlings were marked with unique feather patterns and haphazardly placed in nests, as breeding pairs were in different stages of laying and incubation (Rubin et al., 2021). Parents were removed from the nests once the youngest offspring in a nest gained nutritional independence, which occurs at ∼45 dph. Additionally, birds were visually sexed and separated into isolation cages according to their sex between 55–60 dph, where they continued to receive *ad libitum* access to seed, water, grit, and cuttlefish bone pieces and weekly servings of spinach and egg food with probiotic mixture (Hoffman et al., 2018; Rubin et al., 2021). Each isolation cage held no more than three individuals. After individuals reached sexual maturity (∼95 dph), they were returned to communal flight cages. When individuals were re-captured in adulthood for this study, they were placed back into isolation cages (two individuals per cage, sex separated) for a 2-week acclimation period. Individuals were only disturbed for daily animal care, where they continued to receive *ad libitum* seed, acidified water, egg food mixture, and cuttlefish bone. This experiment utilizes the 22 individuals assigned to the Control incubator (12 female; 10 male), 22 assigned to the Low incubator (11 female; 11 male), and 28 individuals assigned to the Periodic incubator (13 female; 15 male; total of 72 individuals). All procedures listed above were completed at and approved by Auburn University, AL, United States under IACUC #2018-3274.

Beak Coloration Measurements

Beak photographs were taken using an iPhone 8 Plus camera set to standard settings (flash, HDR, Live Mode, Timers, and Filters disabled) within a Table–top photography studio with two light sources on either side of the photography arena. The light sources were marked using lab tape to ensure that light source positioning was consistent across all photos. The camera was secured on a tripod within the photography arena to maintain consistent distances between the bird and the iPhone. Red, blue, and green (RBG) coloration was quantified using ImageJ (Schneider et al., 2012). RGB values were then converted to

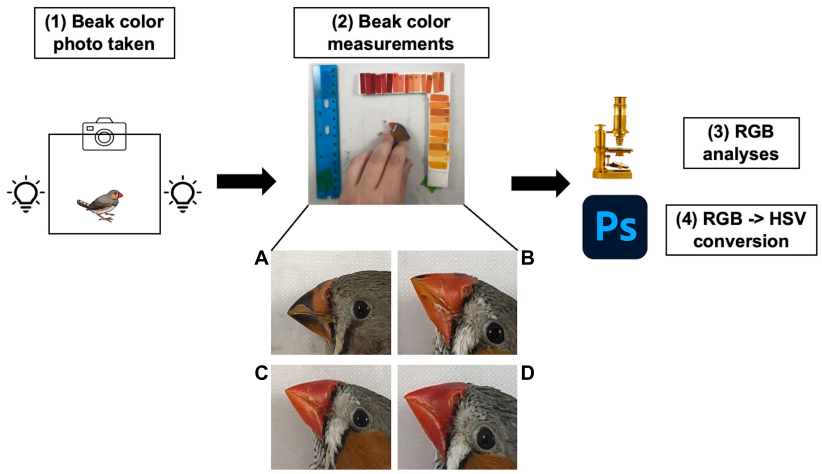

FIGURE 1 | Beak color measurement and RGB/HSV conversion protocols. (1) Beak photos were taken using a standardized photography arena, with lights placed on either side of the arena and a tripod calibrated to a set distance to hold the iPhone. (2) The bird was laid flat onto the surface of the photography arena, ensuring visibility of the beak. Beak color/carotenoid accumulation is visualized in panels **(A–D)**, which represents a male zebra finch at 45 **(A)**, 60 **(B)**, 75 **(C)**, and 95 **(D)** dph. (3) Beak photos were then quantified for red, green, and blue (RGB) measurements using ImageJ, (4) those RGB values were converted into hue, saturation, and value (HSV) correlates and those HSV measurements were cross-examined using Adobe Photoshop.

hue, saturation, and value for each image using an online tool available from RapidTables.[1] Beak color measures were cross-examined and confirmed with Adobe Photoshop CC using the Magic Wand, Histogram, and Color Picker tools with tolerance set to 60 for each photo. To explore the effects of suboptimal incubation temperatures on beak color maturation, measures for hue, saturation, and value were obtained for each bird at 45, 60, 75, and 95 dph (**Figure 1**). Beak coloration was again measured as adults (average age 385.82 ± 32.32 dph) prior to and after a "handling bag test" (described below) to test whether incubation temperature has a long-term effect on beak coloration, to examine a change in beak coloration due to repeated handling, and whether the degree of change is influenced by varying embryonic incubation temperatures. Photographs, ImageJ analyses, and Photoshop analyses were completed by the same individual blind to treatments.

To determine the effect of suboptimal incubation temperature on development and maintenance of beak coloration through an acute stressor, we examined three aspects of color: hue, saturation, and value (HSV). Hue refers to the actual color type, such as red, green, or blue and is measured in degrees on a color wheel, with 0° (and 360°) being red. Birds with lower hues have "redder" beaks, and birds with higher hues have lighter, more orange beaks. Saturation is the intensity (richness) of a color and is measured by a percent, with 0% meaning "no color" and 100% meaning "intense color." Finally, value is the brightness of the color and is also measured by a percent, with 0% being black and 100% being white.

Handling Bag Test

To assess a behavioral stress response to a capture and restraint, we conducted a "handling bag test" adapted from Martin and Réale (2008), illustrated in **Figure 2**. To test the effect of incubation temperatures on behavioral stress responses and maintenance of beak coloration after repeated handling, 72 individuals (36 of each sex) exposed to the Control (n = 12 females; n = 10 males), Low (n = 11 females; n = 10 males), or Periodic (n = 13 females; n = 16 males) incubation regimes were used. Birds were placed in sex-specific cages with two birds per cage (temperature treatments randomized) for a period of 2 weeks, during this time they were not handled and only disturbed to perform daily care. Following this acclimation period, individuals were photographed to document a baseline beak coloration prior to the handling bag test and were then analyzed for their behavioral response to handling stress within 1 min of capture. Individual birds were caught and placed in an opaque brown paper bag suspended in the air with the bird inside for 60 s (Martin and Réale, 2008). During this time, each bird's activity, described as any visible movement or rustling (visible shaking) of the bag during the measurement period, was recorded, and the proportion of time spent moving was calculated. The handling bag test occurred in a separate room from where conspecifics were housed to eliminate potential behavioral responses to chirps and song. An opaque brown paper bag was chosen instead of a mesh bag [as described in Martin and Réale (2008)] to (1) allow for disposal after each individual to prevent olfactory signals between individuals, (2) prevent visual cues while restrained in the opaque bag, and (3) the opaque bag is normally used in our field as a capture and restraint stress protocol. Bag tests were conducted in triplicate for each bird every other day over a period of 5 days, giving birds a day between replicates in which they were not handled and allowed to recover from the stress of capture and restraint. The order of birds that participated in these behavior trials was randomized daily. If two birds shared the same isolation cage, they were not tested in the same day to allow recovery of the individual that was not exposed

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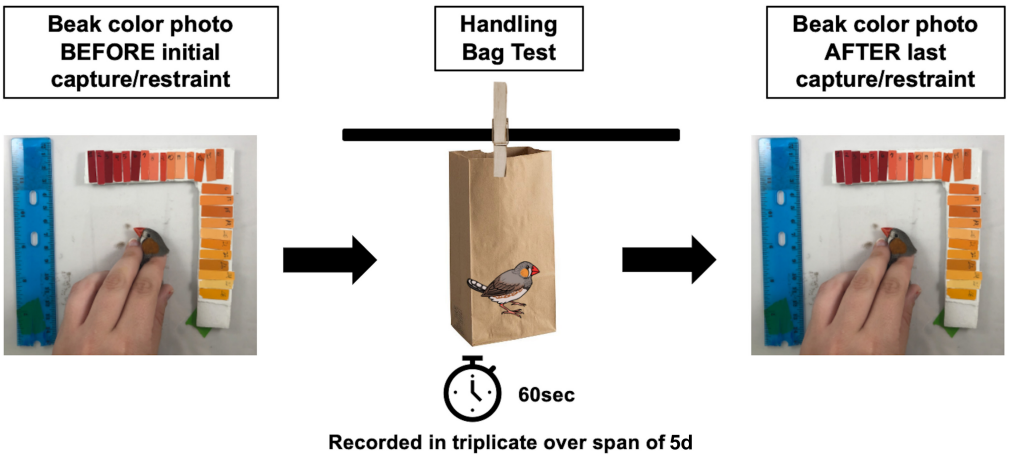

FIGURE 2 | Handling bag test experimental design. Zebra finches at an average age of 386 dph participated in a handling bag test. Prior to the first handling bag test, beak color photos of each bird were taken. Then, each bird was placed in a brown, opaque paper bag and restrained for 1 min, and the time spent moving ("activity,") was recorded. The handling bag test was conducted in triplicate every other day (over span of 5 days) for each bird to ensure for a recovery period. At the conclusion of the third handling bag test, another beak photo was taken.

to the handling bag test in that day. As behavioral responses can vary amongst each trial, three replicates across a period of 5 days were used to generate a "temperament" profile for each bird. In order to measure repeatability of these behavioral responses, we calculated an intra-class coefficient (ICC) to quantify the reliability of phenotypic variation as a fraction of variance among individuals and variance within individuals over time (Hayes and Jenkins, 1997; Bell et al., 2009; Nakagawa and Schielzeth, 2010; Wolak et al., 2012), listed in **Table 3**. The ICC values obtained in this study describe how similar the "time spent moving" replicates are to each other for each individual. Specifically, a value less than 0.5 indicates poor reliability, and a value greater than 0.9 indicates excellent reliability of the data. All handling bag tests, and beak measurements occurred between 08:00 and 09:30 a.m. to ensure that baseline measures were obtained prior to disturbance for daily care.

STATISTICAL ANALYSES

All statistical analyses were performed using RStudio version 4.0.5 "Ghost Orchid" (RStudio Team, 2021) using the lme4 (Bates et al., 2015), tidyverse (Wickham et al., 2019), and lmerTest (Kuznetsova et al., 2017) packages. All error metrics are reported as standard error unless otherwise noted. Aim 1 (beak color changes due to incubation temperature over time) was analyzed using linear mixed effects (LMER) models with a three-way interaction (along with corresponding two-way interactions) between age at measurement, incubation treatment, and sex, with numerical variables being scaled within the model. Additionally, all models for Aim 1 included an independent fixed effect of egg laying order and a random nested effect of nest of origin and individual to account for both genetic and individual differences. When interactions were significant in the global model ($P < 0.05$), those variables were analyzed separately. Hue, saturation, and value were used as measures of beak color. We used principal component analysis (PCA) to reduce collinearity between these variables. PCA showed that one component had an eigenvalue of 2.01, representing 67% of the variance in the data (**Supplementary Figure 2** and **Supplementary Table 5**). When alternative models were performed, consistent results were obtained. Therefore, we are presenting hue, saturation, and value parameters separately.

Aim 2 (observing short-term changes in beak color from the handling bag test) was analyzed using LMER with a three-way interaction (along with corresponding two-way interactions) between sex, treatment, and timepoint (before/after the handling bag test), and a random effect of individual.

For Aim 3 (determining if thermal stress alters future responses to a stressor), we used a general linear model (lm) with fixed effects of treatment and sex and an interaction between the two variables. The independent variable in this case was the proportion of time spent moving while restrained in the bag. To verify the reliability of these repeated behavioral measures in Aim 3, we calculated an intra-class coefficient (ICC) using a generalized linear mixed effects model (binomial) with ID as a random effect using the rptR package (Stoffel et al., 2017). Statistical summaries are reported in **Tables 1–4**. A comprehensive table of estimates for each model are reported in **Supplementary Tables 1–3**.

RESULTS

Aim 1: Effects of Suboptimal Incubation Temperature on Beak Coloration Throughout Development

All statistical measures for Aim 1 can be found in **Table 1** and **Supplementary Table 1**. A graphical summary of the data can be found in **Figure 3**. We found that individuals in the

TABLE 1 | Statistical summary of effects of incubation temperature on beak color before and after sexual maturity (Aim 1) using a global linear mixed effects model.

Independent variable	Variance due to egg ID (random)	Residual variance	Fixed effect	*df*	*F*-value	*P*-value
Hue (°)	1.178; SD = 1.085	16.40; SD = 4.05	Treatment	2	0.1623	0.8502
			Sex (Male)	**1**	**201.9**	**<0.001*** **
			Age	**1**	**181.6**	**<0.001*** **
			Lay order	1	0.2158	0.6446
			Treatment*Sex	**2**	**3.158**	**0.0438***
			Treatment*Age	2	0.5964	0.5514
			Sex*Age	**1**	**19.77**	**<0.001*** **
			Treatment*Sex*Age	2	1.273	0.2813
Saturation (%)	0.6689; SD = 0.8179	102.68; SD = 10.13	Treatment	2	0.0273	0.9731
			Sex	1	0.1841	0.6682
			Age	**1**	**14.65**	**<0.001*** **
			Lay order	**1**	**4.912**	**0.0339***
			Treatment*Sex	2	0.1813	0.8343
			Treatment*Age	2	0.1548	0.8567
			Sex*Age	1	1.733	0.1888
			Treatment*Sex*Age	2	0.6211	0.5379
Value (%)	0.00; SD = 0.00	109.4; SD = 10.46	Treatment	2	0.2037	0.8158
			Sex	**1**	**19.56**	**<0.001*** **
			Age	**1**	**12.12**	**<0.001*** **
			Lay Order	1	2.416	0.1209
			Treatment*Sex	2	1.636	0.1963
			Treatment*Age	2	0.1295	0.8786
			Sex*Age	**1**	**6.671**	**0.0102***
			Treatment*Sex*Age	2	0.6189	0.5391

Statistical significance is indicated by asterisk(s) (*) and are bolded where Pr ≤ 0.05. Hue is measured in degrees (°), and both saturation and value are measured as percentages (%). Numerical variables (i.e., Age and Egg Order) were scaled within the model. Interactions are specified within the "Fixed effect" column by a single asterisk (*) between variables.

Periodic group had significantly lower beak hues than those in the Control group [1.61° ± 0.734; $t_{(371.00)}$ = 2.19; P = 0.029]. Males also exhibited significantly lower beak hues than females [7.59° ± 0.825; $t_{(329.14)}$ = 9.20; $P < 0.001$]. We found a significant interaction between sex and age [−2.70 ± 0.753; $F_{(1,348.65)}$ = 19.77; $P < 0.001$]. When sexes were analyzed separately, we found that male hue decreased as they aged [3.84° ± 0.64; $t_{(1932.00)}$ = 6.00; $P < 0.001$, **Figure 4**]. In females, we found that female hue decreased with age [1.19° ± 0.379; $t_{(145.15)}$ = 3.14; P = 0.002, **Figure 4**].

In contrast to hue, we found no significant interaction between sex and age on beak saturation. However, age significantly affected saturation, where beak saturation (intensity of color) increased by 2.77% ± 1.28 [$t_{(355.43)}$ = 2.17; P = 0.031] in both sexes (**Figure 4**) and plateaued around 75 dph. We did not find a significant effect of incubation treatment on neither male nor female beak saturation. Interestingly, we found that as egg laying order increased, saturation levels decreased [1.22% ± 0.552; $t_{(31.97)}$ = 2.22; P = 0.0339].

Males exhibited significantly lower beak values than females [5.92% ± 1.98; $t_{(372.00)}$ = 2.99; P = 0.003]. There was a significant interaction between sex and age on beak value [$F_{(1,372)}$ = 6.67; P = 0.010], so sexes were analyzed separately. There was no significance with regard to males. However, we found that female beak saturation increased [3.84% ± 1.43; $t_{(179.00)}$ = 2.69; P = 0.0078] with age.

Aim 2: Effects of Repeated Handling and Influence of Suboptimal Incubation Temperatures on Beak Coloration

Generally, we found that hues in all individuals were higher prior to the initial handling bag test compared to after the final bag test in the global model [1.0833° ± 0.3754; $t_{(65.71)}$ = 2.89; P = 0.0053], but this is dependent on sex [$F_{(1,66)}$ = 17.20; $P < 0.001$]. We also found a sex x treatment interaction [$F_{(2,76)}$ = 3.46; P = 0.036]. When sexes were analyzed separately, we found that males in the Periodic treatment had significantly higher hues [1.55° ± 0.77; $t_{(43.52)}$ = 2.02; P = 0.0496] than Control males. We found a significant interaction between sex x timepoint ($P < 0.001$). Females had significantly higher hues [1.08° ± 0.40; $t_{(33.00)}$ = 2.70; P = 0.011] before the handling bag test regardless of the incubation treatment, meaning that female beak color became redder after repeated handling. In contrast, male beak hue did not change with repeated handling [$t_{(32.82)}$ = 1.58; P = 0.124] (**Figure 5A**). We feel that it is important to mention that individuals in both the Periodic and Low treatments tended to have lower hues than Control birds [Periodic: $t_{(73.32)}$ = 1.91; P = 0.064; Low: $t_{(73.32)}$ = 1.67; P = 0.099, respectively].

We found a statistically significant sex x timepoint interaction [$F_{(1,66)}$ = 50.12; $P < 0.001$] when analyzing beak saturation. When sexes are analyzed separately, we found that males had significantly increased beak saturation following the last handling

TABLE 2 | Statistical summary of the short-term changes in beak color due to repeated handling and incubation temperature (Aim 2) using a global linear mixed effects model.

Independent variable	Variance due to bird ID (random)	Residual variance	Fixed effect	*df*	*F*-value	*P*-value
Hue (°)	7.890; SD = 2.809	0.846; SD = 0.920	**Sex**	**1**	**191.7**	**<0.001*****
			Treatment	2	0.8195	0.4445
			Timepoint	1	0.0777	0.7813
			Sex*Treatment	**2**	**3.463**	**0.0363***
			Sex*Timepoint	**1**	**17.20**	**<0.001*****
			Treatment*Timepoint	2	1.648	0.2003
			Sex*Treatment*Timepoint	2	0.5186	0.5977
Saturation (%)	10.22; SD = 3.197	6.26; SD = 2.50	Sex	1	0.1690	0.6824
			Treatment	2	0.2723	0.7624
			Timepoint	1	0.2983	0.5868
			Sex*Treatment	2	1.510	0.2279
			Sex*Timepoint	**1**	**50.12**	**<0.001*****
			Treatment*Timepoint	2	0.0410	0.9600
			Sex*Treatment*Timepoint	2	2.7820	0.06915
Value (%)	6.553; SD = 2.56	5.11; SD = 2.26	**Sex**	**1**	**194.9**	**<0.001*****
			Treatment	2	0.1339	0.8749
			Timepoint	**1**	**4.415**	**0.0394***
			Sex*Treatment	**2**	**8.362**	**<0.001*****
			Sex*Timepoint	1	0.4459	0.5066
			Treatment*Timepoint	2	0.9785	0.3812
			Sex*Treatment*Timepoint	2	0.2600	0.7719

Statistical significance is indicated by asterisk(s) (*) and are bolded where Pr ≤ 0.05. Hue is measured in degrees (°), and both saturation and value are measured as percentages (%). Interactions are specified within the "Fixed effect" column by a single asterisk (*) between variables.

TABLE 3 | Intra-class coefficient (ICC) calculations for repeated capture and restraint of zebra finches.

	ICC	Reliability
All (sexes and treatments combined)	0.3010	Poor
Females (all treatments)	0.6028	Moderate
Males (all treatments)	0.5561	Moderate
Females (Control)	0.7031	Moderate
Males (Control)	0.3727	Poor
Females (Periodic)	0.6596	Moderate
Males (Periodic)	0.6729	Moderate
Females (Low)	0.3396	Poor
Males (Low)	0.4106	Poor

An ICC value of 0.5 or below indicates poor reliability of the data, 0.5–0.75 moderate reliability, 0.75–0.9 good reliability, and >0.9 excellent reliability. These ICC values were calculated using a binomial LMER.

TABLE 4 | Statistical summary elucidating the effects of suboptimal incubation temperature on activity levels (Aim 3) using a global linear model (LM).

Independent variable	Fixed effect	*df*	*F*-value	*P*-value
AvgProp	Treatment	2	0.5123	0.6015
	Sex	1	0.1183	0.7320
	Treatment*Sex	2	2.8405	0.0656†

Statistical significance is indicated by asterisk(s) (*) where P ≤ 0.05. P-values trending toward statistical significance are indicated by a dagger symbol (†). The independent variable is "AvgProp," which is the average proportion of time spent moving while restrained during the handling bag test.

bag test [3.88% ± 1.19; $t_{(32.95)}$ = 3.26; P = 0.003; **Figure 5B**]. On the other hand, females had significantly decreased beak saturation following the last handling bag test [3.62% ± 0.95; $t_{(33.00)}$ = 3.80; P = 0.001; **Figure 5B**].

We found that males had significantly lower beak value (intensity) when compared to females in the global model [13.25% ± 1.46; $t_{(100.40)}$ = 9.06; $P = 1.1 \times 10^{-14}$; **Figure 5C**]. Additionally, a sex x treatment interaction was observed [$F_{(2,69)}$ = 8.36; $P < 0.001$], so sexes were analyzed separately. Compared to Control males, males in the Low group trended toward higher beak values [$t_{(57.89)}$ = 1.92; P = 0.060], and males in the Periodic group exhibited significantly higher beak values [3.54% ± 1.45; $t_{(57.32)}$ = 2.44; P = 0.018]. Females in the Periodic group exhibited significantly lower beak values [2.74% ± 1.30; $t_{(42.77)}$ = 2.10; P = 0.041] than females in the Control group. All statistical measures mentioned above can be found in **Table 2** and **Supplementary Table 2**.

Aim 3: Fluctuating Incubation Temperature Alters Future Responses to a Handling Stressor

We estimated an ICC of 0.301 (95% CI: 0.080–0.432) for whether an animal moves during the one-minute stress test, indicating that the data is poorly reliable in quantifying an overall behavioral profile for each individual when both sexes and all treatments are considered. This ICC was calculated based on a binomial model, meaning that the data was coded by

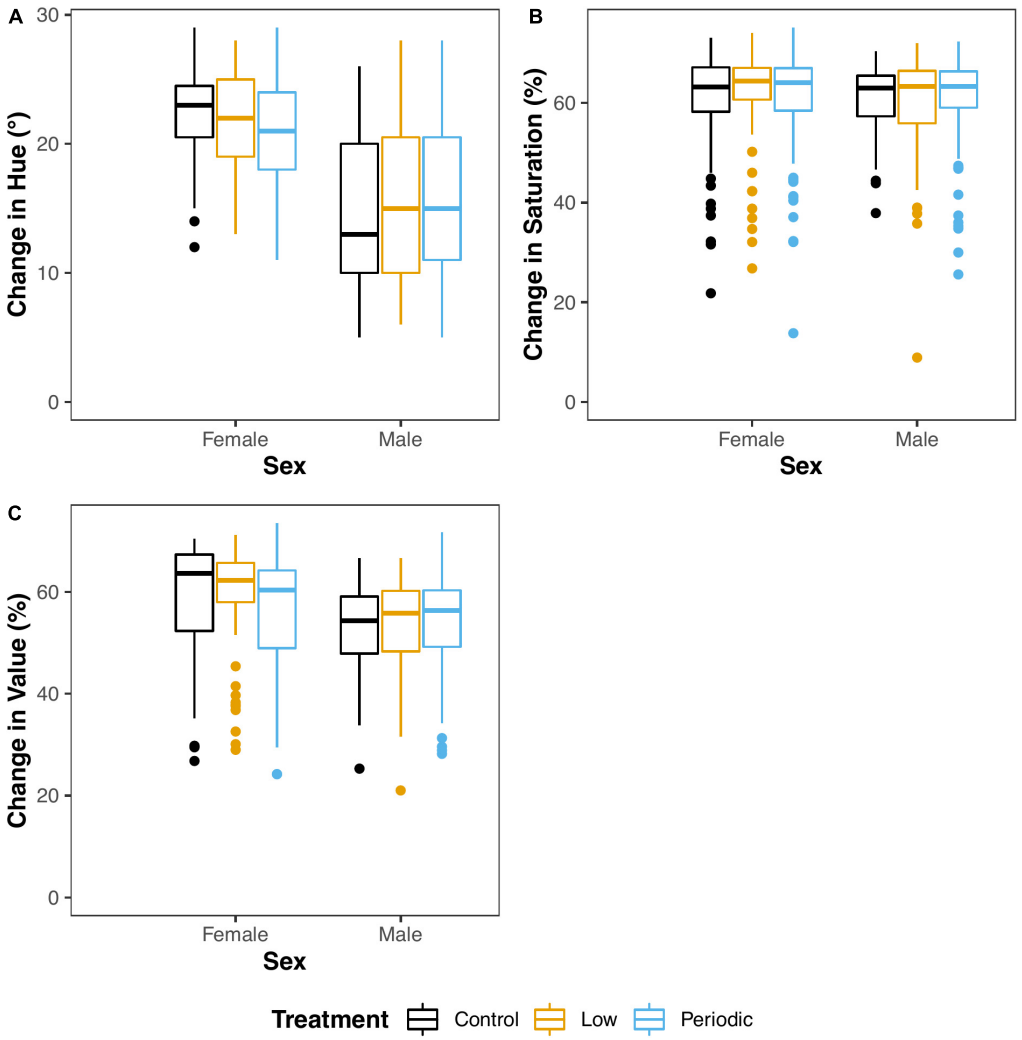

FIGURE 3 | Suboptimal embryonic incubation temperatures do not impact beak color (hue, saturation, value) development. Control, Low, and Periodic treatments are shown in black, yellow, and blue, respectively. The changes in hue **(A)**, saturation **(B)**, and value **(C)** are measured on the y-axis, measured in degrees and percentage, respectively. Sex is indicated on the x-axis.

specifying whether the animal moved during the restraint period. ICCs were then calculated for each sex and treatment, which are listed in **Table 3**. Link- and original-scale approximation bootstrap repeatabilities for ID when calculating ICCs are listed in **Supplementary Figure 1** and **Supplementary Table 4**. We found that males tended to elicit less activity (proportion of time spent moving) than females when restrained during the handling bag test [$t_{(66.00)} = 1.98$; $P = 0.051$]. We did find a sex x treatment interaction trending toward statistical significance [$F_{(2,66)} = 2.84$; $P = 0.066$], so we analyzed sexes separately due to biological significance. Males in the Low treatment group showed relatively higher activity levels than males in the Control group [0.26 ± 0.10; $t_{(33.00)} = 2.48$; $P = 0.018$, **Figure 6**], but there was no effect of the Periodic group on activity levels in males [$t_{(33.00)} = 1.10$; $P = 0.28$]. The incubation temperature treatment had no effect on activity levels in females. Statistical measures are summarized in **Table 4** and **Supplementary Table 3**.

DISCUSSION

Our study sheds light on the impacts of suboptimal embryonic incubation temperatures on beak coloration and the behavioral stress response in zebra finches throughout post-hatch development and into sexual maturity. Because fluctuating incubation temperatures are commonly experienced by avian eggs, in nature we sought to disentangle the effects of temperature variation from absolute temperature and to illuminate their downstream effects on beak color development using zebra finches as a model. We predicted that females in the Low and Periodic groups would decrease beak hue later in life, as females have been shown to be the most susceptible to environmental stressors. Because of this, we also predicted that females would exhibit higher activity levels during capture and restraint while birds from the Periodic group show improved stress tolerance due to frequent exposure

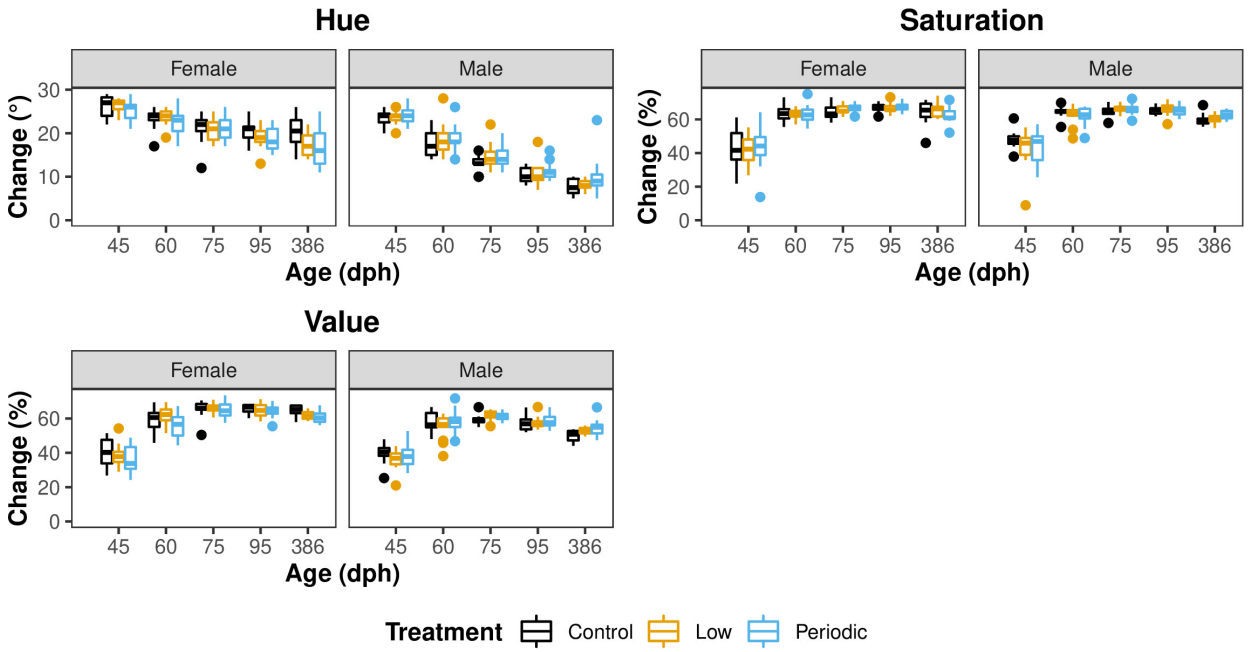

FIGURE 4 | Male and female hue decreases over time, and saturation and value plateau on 60 and 75 dph, respectively. Females are shown in the left panel, and males are shown in the right panel. Control, Low, and Periodic treatments are shown in black, yellow, and blue, respectively. The changes in hue, saturation, and value are measured on the y-axis, measured in degrees and percentage, respectively. Age is indicated on the x-axis, including 45, 60, 75, 95, and 386 dph, respectively.

to a mild stressor during development. In sum, we did not find an overall effect of incubation temperature on beak color development, but we found that periodically cooled females had decreased hues at 386 dph compared to Control females at that age. We also found that eggs laid later within a clutch had lower beak saturation than earlier laid eggs. When these individuals were subjected to repeated capture and restraint, females had lower beak hue and saturation following the final handling bag test. Conversely, we found that males had increased beak saturation following the final handling bag test. Lastly, we found that males within the Low incubation treatment had relatively higher activity levels than Control males when restrained.

Embryonic Incubation Temperature Had a Stronger Effect on Female Beak Color Maturation Than in Males

Many studies have explored the environmental, anthropogenic, and pathogenic effects of beak color in avian species. Beak color in goldfinches (*Spinus tristis*) is similarly regulated by carotenoid pigments and is impacted by repeated handling stress and immunostimulatory lipopolysaccharide (LPS) injection, as yellow saturation decreased within 6.5 h of repeated capture, and upon addition of LPS injection, beak hue and luminance drastically decreased (Rosenthal et al., 2012). Additionally, beak color in birds has been extensively experimentally modified via eliciting a stress response, modifying diet, or immune activity over a time period of weeks (Blount, 2003; Ardia et al., 2010; Rosenthal et al., 2012). Thus, it is apparent that maintaining beak color through the context of carotenoid distribution is a major tradeoff at the expense of reproduction (Bertrand et al., 2006). It is important to note that many studies that analyze beak color mainly use hue as a parameter, as hue is the main component of defining a particular color. However, data are lacking for other aspects of beak color such as saturation and value and so the consequences of changes in these traits on sexual ornamentation and reproduction is not well-understood.

First, we found that females at 386 dph had lower beak hues than Control females. This finding aligns with our prediction that females would be more affected than males, but the "attractiveness" of a redder beak in a female remains unclear without conducting mating trials. Specifically, females prefer males with the reddest, brightest beak color (Blount, 2003; Burley and Coopersmith, 2010; Merrill et al., 2016). Conversely, males prefer females whose beaks are not too red or too yellow but more orange with less intense coloration (Zann, 1996; McGraw, 2006). A previous study found that male and female zebra finches subjected to daily, 10-min handling treatments for 4 weeks displayed deeper orange/red beak coloration than control animals (McGraw et al., 2011). Although "attractiveness" of a redder beak on a female to a male beak is relatively unknown, a small set of evidence shows that males who experience no known stressor during development prefer females with orange beaks (higher hues) (Zann, 1996; McGraw, 2006). If a males' preference is not influenced by a developmental stressor, high carotenoid allocation resulting in lower beak hue in a female may not be beneficial, as

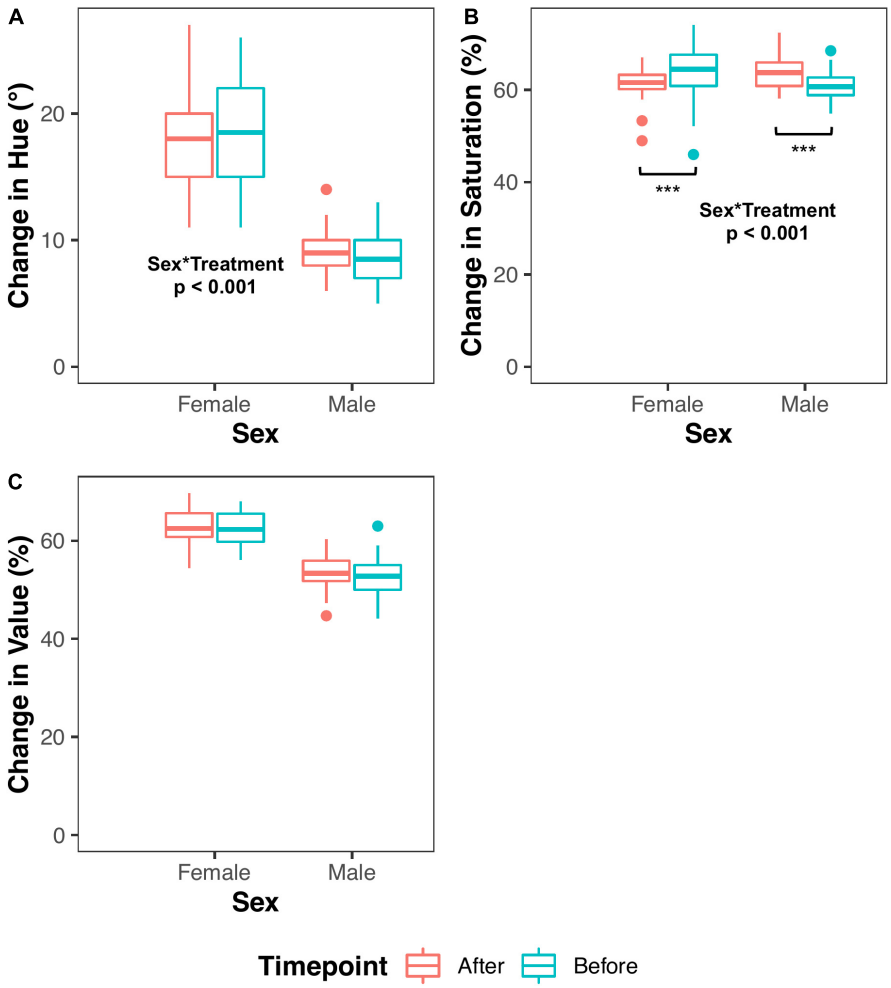

FIGURE 5 | Beak hue and saturation are impacted by repeated capture and restraint. The change in the beak color parameter (hue, saturation, or value) is presented on the y-axis, and sex is shown on the x-axis. Data indicated in red boxplots are indicative of "Before" the handling bag test, whereas blue boxplots are indicative of "After" the handling bag test. Significant sex*timepoint interactions are mentioned where applicable. **(A)** Female beak hue significantly decreases following the initial handling bag test. **(B)** Both male and female beak saturation are inversely affected following the initial handling bag test. **(C)** There are no effects of the handling bag test on beak value. Three asterisks (***) indicate $P \leq 0.001$.

males may interpret this as a more "masculine" phenotype. Previous studies have shown that exogenous testosterone administration increases beak redness (lower hue) in female zebra finches (McGraw, 2006). Since testosterone increases redness in beaks but also suppresses immune functions, testosterone can mediate a trade-off between attractiveness and immune function. To fully understand these dynamics, future studies should evaluate how consistently low and fluctuating incubation temperature affects sex steroid levels, attractiveness via mate choice trails, and immunocompetence. It is possible that consistently low or periodic cooling events that occur during embryonic incubation of the parental generation may increase testosterone levels within the yolk of subsequent offspring (Gil et al., 2004). McGraw (2006) proposes that testosterone-induced immunosuppression in females with naturally high testosterone levels may divert carotenoids away from the beak and thus fading in beak color.

In contrast to females, we found that there was no significant effect of suboptimal embryonic incubation temperatures on male beak hue maturation. It is possible that males were able to maintain their beak hue despite embryonic perturbation as their post-hatch environment contained *ad libitum* access to food and nutrients. While there is strong evidence that an individual's secondary sex characteristics can reflect developmental history (Merrill et al., 2016) especially in birdsong (Buchanan et al., 2003; Spencer et al., 2005), beak hue does not appear to be a strong indicator of developmental history with regard to male zebra finches. It has been shown that carotenoid-derived beak color in males is sex-steroid-dependent (e.g., androgens) (Nelson, 2005), where maternally-deposited androgens within the yolk increase offspring competitiveness and development (Schwabl, 1996). Female red-legged partridges (*Alectoris rufa*) have been found to mate with redder males and produce more eggs which could favor the survival of later hatchlings by increasing androgen

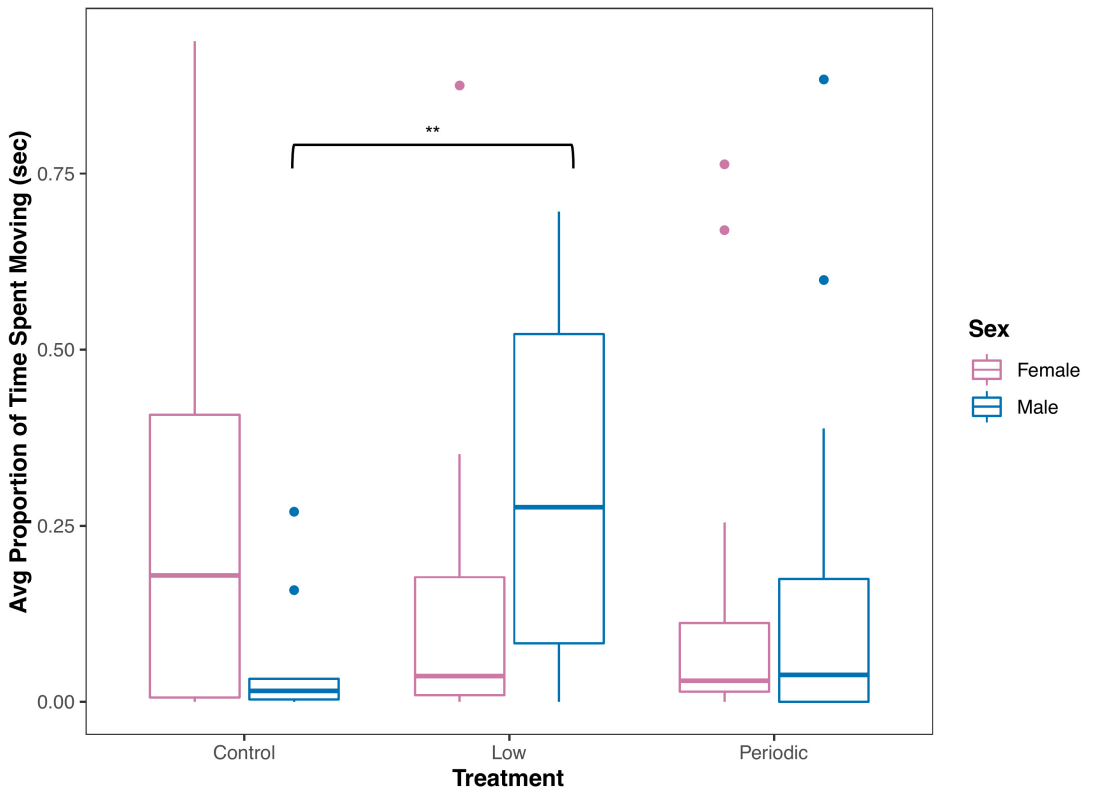

FIGURE 6 | Males in the Low incubation treatment had higher activity levels than those in the Control treatment. The average proportion of time (seconds) spent moving during the handling bag test is recorded on the y-axis. The three incubation treatment groups are shown on the x-axis (Control, Low, and Periodic). Females are indicated by pink boxplots, and males are indicated by blue boxplots. Two asterisks (**) indicate $P \leq 0.05$.

allocation to eggs (Alonso-Alvarez et al., 2012). The next step would be to quantify circulating androgen levels to determine whether an ability to secrete comparable levels of androgen would explain how males from eggs incubated at Low or Periodic regime can maintain beak hue later in life.

We did not observe an effect of suboptimal incubation temperature on beak saturation in males nor females. However, we did confirm that beak saturation (color richness) increases with age in both sexes. Interestingly, we found that eggs laid later in a clutch had decreased beak saturation compared to earlier-laid eggs. It has previously been discovered that yolk testosterone levels increase as laying order increases in canaries and zebra finches (Schwabl, 1993), which could have implications of yolk androgen levels and its impacts on beak saturation later in life. This pattern can vary depending on other factors, including diet. When mothers were fed a low-quality diet, their yolk testosterone levels decreased with egg laying order (Sandell et al., 2007). Therefore, it is possible that the eggs utilized in this experiment that were laid later in the clutch had higher levels of testosterone by the mother, resulting in decreased beak saturation later in life. This is a loose speculation, as the parents of eggs from this experiment were not experimentally monitored nor exposed to any known stressors to cause variations in testosterone supplementation into the yolk.

Repeated Capture and Restraint Has Sex-Specific Effects on Beak Hue, Saturation, and Value

We found that females decreased beak hue and saturation while males increased beak saturation following the handling bag test. These findings are similar to a previous study where zebra finches of both sexes were subjected to daily handling stress over 4 weeks, and stressed males lost body mass and marginally decreased circulating carotenoid concentrations (McGraw et al., 2011). However, our results in females differed from the aforementioned study, where stressed females maintained their orange beak color. Both McGraw et al. (2011) and the current study show a sex-specific effect on handling stress on beak coloration in zebra finches, suggesting that males and females experience differential prioritization of beak color. It has been shown that carotenoids can be allocated to various parts of the body, such as within the integument, retina, and liver tissue (Rowe et al., 2012). Specifically, carotenoids can be stored within liver tissue until needed during periods of molt or migration (Negro et al., 2001).

It is possible that, because of repeated capture and restraint, females mobilized carotenoid pigments, whereas males pulled carotenoid pigments from their beak tissue. The current study exposed finches to a handling stressor every other day for 5 days. Since this is much shorter and less frequent exposure than daily handling over 4 weeks used in McGraw et al. (2011), the data in the current study demonstrate that beak coloration can change with relatively mild handling stress. It is known that beak hue can change rapidly in response to a short-term stressor, as glucocorticoid-related stress impairs various condition-dependent traits (Buchanan, 2000). Generally, color saturation describes the vividness, richness, or intensity of a color. In this context, females had 'less vivid' beaks, whereas males had "more vivid" beaks because of the handling bag test. Males subjected to suboptimal incubation temperatures (both Low and Periodic groups) exhibited significantly higher beak values (brighter) compared to Control males following the capture and restraint protocol. It has been found that repeated handling significantly decreases body mass and depletes circulating carotenoid levels during the course of handling, but not long-term (McGraw et al., 2011). It is possible that short-term perturbation of carotenoids within beak tissue may make the beak itself less pigmented, thus affecting beak value, but our results show that individuals can potentially recover beak coloration by re-depositing carotenoid pigments within the beak. Unfortunately, we did not collect body mass/condition measures at or around the time of behavioral testing. However, our *post-hoc* testing of our previous study revealed that 10 dph individuals in the Periodic group were 14.2% lighter than individuals in the Low and Control groups (Rubin et al., 2021). Therefore, these results may be snapshots of the short-term changes in beak saturation because of repeated handling showing the movement of physiological and energetic resources away from secondary sex characteristics.

Embryonic Incubation Treatment Alters Future Responses to a Capture and Restraint Stressor

Overall, we found that males exhibited lower activity levels than females when restrained during the handling bag test. This is to be expected, as females generally elicit a more heightened stress response than males (Martins, 2004; Verhulst et al., 2006; Spencer et al., 2010; Marasco et al., 2012), which has been observed in rats in response to alcohol (Rivier, 1993), but females have been found to better cope with chronic stressors over time (Dalla et al., 2005). However, females do not always respond greater than males, as a previous study found that there is no sex differences in the CORT response to handling stress in 16 dph or as adults (Wada et al., 2008). However, we did find that males in the Low incubation group exhibited higher activity levels than males in the Control group. While the embryonic environment on the behavioral development of birds remains relatively unexplored, a recent study has shown that chronic exposure to suboptimal temperatures of 27.2°C for 1 h twice a day resulted in experimental chicks with elevated neophobic responses in novel food and novel environment tests, and these experimental chicks also showed higher corticotropin-releasing factor in the nuclei of the amygdala, which is involved in regulating fear-related behaviors (Bertin et al., 2018). Chronic exposure to low incubation temperatures has been shown to cause oxidative damage and changes in antioxidant pathways (Loyau et al., 2014; Bertin et al., 2018), which may have impacted the developmental programming of behavioral responses observed in males during the handling bag test. In fact, a study utilizing the same cohort of animals used in the present study found that individuals in the Periodic and Control groups were able to habituate, in terms of their adrenocortical response, to a repeated capture and restraint stress, whereas the individuals exposed to the Low incubation treatment were not (Rubin et al., 2021). This lack of the ability to habituate to a repeated stressor in the Low group viewed in conjunction with higher activity levels during restraint from the present study suggest a possible neuroendocrine link between heightened activity and elevated CORT levels. This inability to habituate to a subsequent stressor can lead to higher circulating CORT levels throughout life, which negatively impacts fitness-related traits, such as immunocompetence, growth, and survival (Sapolsky et al., 2000; Wada et al., 2015; Jimeno et al., 2018; Rubin et al., 2021). These hormonal responses are dependent on neuroendocrine pathways that are mostly established during early life. Lastly, there were no long-term effects of incubation temperature observed in females. Overall, these data suggest that incubation temperature and variability may contribute to the stress responses mounted later in life, which can have downstream effects on beak coloration and attractiveness.

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 reviewed and approved by Auburn University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MC and AR carried out the experiment and collected the data. MC wrote the manuscript, analyzed the data, and generated graphical representations of the data. HW assisted with manuscript writing, proofreading, and statistical analyses. All authors designed the study, contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.901303/full#supplementary-material

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Food Restriction Reveals Individual Differences in Insulin-Like Growth Factor-1 Reaction Norms

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Most organisms have to cope with unpredictable environmental challenges such as fluctuations in nutritional resources. Insulin-like growth factor-1 (IGF-1) is an evolutionarily conserved hormone that is highly sensitive to the individual nutritional status and regulates major life-history traits including lifespan and reproduction across vertebrates. We investigated the role of IGF-1 during periods of food shortages by altering between two feeding regimes (110 and 70% of daily food intake) after a period of *ad libitum* feeding in captive bearded reedlings (*Panurus biarmicus*). Each dietary treatment was repeated twice. Birds lost mass under food restriction, but the magnitude of mass change depended on the preceding dietary conditions. Moreover, bearded reedlings showed large, repeatable individual differences in their IGF-1 reaction norms with some individuals increasing IGF-1 levels in response to a restricted diet, whereas others showed no responses or decreased IGF-1 levels. This variation was explained by differences in average body mass: heavier individuals had higher IGF-1 levels during the control treatment and were more likely to decrease IGF-1 levels in response to the dietary restriction than did lighter ones. This result uncovers an individual by environment interaction (I × E) and may have important implications for the evolution of IGF-1 related hormonal phenotypes in this species.

Keywords: IGF-1 (insulin-like growth factor-1), stress, *Panurus biarmicus*, body mass, endocrinology, nutrition

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INTRODUCTION

Unpredictable fluctuations in the availability of food (e.g., because of droughts, cold winters) are ubiquitous and organisms have developed an array of morphological, physiological and behavioral adaptations to cope with such environmental challenges (Groscolas and Robin, 2001; Harshman and Zera, 2007; Killen et al., 2011). The role of the endocrine system is particularly interesting in this context, because hormones are highly plastic traits that integrate external (environmental) and internal (e.g., nutritional status) information to respond to stochastic environments (Harshman and Zera, 2007; Regan et al., 2020). Across their responsiveness, hormones show a reaction norm (i.e., differential phenotypic expression of a given genotype due to changing environments), which is highly variable within and among individuals (Pigliucci, 2001;

Cockrem, 2013). The among-individual variation in reaction norms (individual by environment interaction, I × E) shape phenotypically plastic responses to resource availability in a given environment (Williams, 2008; Baugh et al., 2014; Lendvai et al., 2014; Hau and Goymann, 2015; Madliger and Love, 2016; Vitousek et al., 2018; Houslay et al., 2019). The degree of plasticity influences not only the individual but also the ability of the populations to respond to small immediate changes, such as food shortages or predator attacks, and to long-term effects, such as climate change (Reed et al., 2006; Visser, 2008).

One of the hormonal systems that evolved to respond to the variation in food availability is the insulin/insulin-like signaling pathway (IIS) (Regan et al., 2020). IIS is known to have a prominent role in regulating energy metabolism and is directly integrated with nutrient-sensing cellular mechanisms (Saltiel and Kahn, 2001). In vertebrates, the main ligand of this system is insulin-like growth factor-1 (IGF-1), one of the key factors regulating the organism's metabolism and development in relation to its nutritional status (Dantzer and Swanson, 2012; Lodjak and Mägi, 2017). These effects might be tightly linked to how IGF-1 determines the transition from the catabolic to the anabolic state. Low nutrient/energy availability is suggested to decrease IGF-1 production and secretion, which leads to increased cell recycling, autophagy, and apoptosis (Bitto et al., 2010; Adler and Bonduriansky, 2014). These processes can have direct and indirect fitness effects by down-regulating reproduction, growth, and the immune response (Wang and Levine, 2010; Gao et al., 2019). On the other hand, up-regulation of IGF-1 can delay muscle atrophy (i.e., an excessive amount of apoptosis of cells) during food restriction and reduce overall weight loss (O'Sullivan et al., 1989; Cleveland et al., 2009; Abe et al., 2019).

Even though fluctuations in food availability frequently occur in nature and IGF-1 might facilitate adaptations toward these conditions (O'Sullivan et al., 1989), our knowledge about the role of IGF-1 in shaping responses to such events in wild animals is limited. The majority of literature presents the findings of medical and agricultural sciences, focusing on humans and laboratory or farmed animals (Robinson et al., 2006; Berryman et al., 2008; Valente et al., 2013; Mauch et al., 2016; Rahmani et al., 2019). There are only a few examples from wild, free-living animals, such as a study in Eastern fence lizard (*Sceloporus undulatus*) which shows that food-restriction decreased plasma IGF-1 levels (Duncan et al., 2015). Another study on nestlings of a passerine species discusses the possibility that the growth-enhancing effects of IGF-1 during early development might be affected by parental food supply (Lodjak et al., 2014). Hence, to understand the variation in IGF-1 responses to current food availability and its contribution to shaping physiological phenotypes, it is important to aim for experimental studies on wild-type animals originating from natural populations.

To explore the role of the IGF-1 response to variation in food availability in a wild animal, we conducted a food-restriction experiment, using captive adult bearded reedlings (*Panurus biarmicus*). We repeatedly exposed individuals to changing dietary regimes, by alternating between control (110% of the daily intake) and restricted diet (70% of the daily intake). After each dietary treatment, we measured body mass and circulating IGF-1 levels of the birds. Our experimental design allowed us to disentangle among- and within-individual hormonal variation and to make predictions at both levels. Considering that IGF-1 levels are regarded to reflect the individual nutritional status, we made two predictions. First, we predicted that among individuals, larger (heavier) birds would have higher circulating IGF-1 levels. Second, we predicted that within individuals, circulating IGF-1 levels would decrease in response to the restricted dietary regime and this decrease would be proportional to body mass loss under the restricted diet.

MATERIALS AND METHODS

General Methods

In July 2017, we caught 24 Bearded reedlings (*Panurus biarmicus*) at Hortobágy-Halastó, (47°38'13.7 N and 21°04'42.8 E, Hungary) using mist-nets. At the time of capture, all birds were juveniles (yearlings). The age and sex were determined by examining plumage and bill coloration (Svensson, 1992). All birds were ringed with a standard aluminum ring, and we measured tarsus length (to the nearest 0.01 mm) and body mass (to the nearest 0.1 g).

Immediately after the capture and the subsequent measurements, the birds were transferred into an outdoor aviary (3.65 × 3.35 × 2.75 [L × W × H] m) located at the University of Debrecen (47°33'32.9 N and 21°37'14.6 E). The aviary was furnished with reed bundles, branches, and a pool (1 m^2 water surface). Food (a mixture of grated apple, carrot, quark, a commercial soft food mixture for insectivorous birds, ground dry cat food as a protein supplement and live mealworms) and water were provided *ad libitum*. The birds remained for seven months in the aviary before the onset of the experiment (5 February 2018) to habituate to the captive conditions. By the time the experiment started, all individuals had completed their post-juvenile molt and the reproductive period had not yet begun.

We followed all applicable international, national, and institutional guidelines for the use of animals. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and approved by the Regional government agency (license no HBB/17/00870-3/2015).

Experimental Protocol

The experimental protocol was based on Lendvai et al. (2014), with modifications. Two weeks prior to the experiment, the birds were captured, weighed and transferred into individual cages (measuring 30 × 26 × 32 [L × W × H] cm), located in the outdoor housing facilities.

During the acclimation period, individuals received *ad libitum* food (as described above) and water. Mealworms were not provided, because live mealworms can leave the feeder, which could have affected the dietary treatments (see below). After 2 weeks of acclimation in the individual cages, we measured the daily food intake (DFI) for each individual for five consecutive days. We fed the birds every morning between 09:00 and 10:00

by filling their feeder with 38 g of food. A 24 h later, we removed the bottom tray and the feeder from each cage and measured the weight of the remaining, and spilled food with a digital scale. The DFI was calculated as the difference between the initial weight in the feeder and the weight of remaining food plus spillage. This procedure was performed twice over 2 weeks, and the average daily food intake (ADFI) was calculated as the average of 10-day DFI values for each individual.

After measuring the food intake, birds were kept under *ad libitum* food regime for one additional week. After this period, individuals were randomly assigned to one of the treatment groups: food-restricted (70% of their individual ADFI) or control (110% of their individual ADFI). The treatment was based on previous studies showing that a 30% reduction in food is sufficient to trigger changes in hormone levels (e.g., Valle et al., 2015, 2020). The *ad libitum* diet differs from the control diet in that it is available in large quantities without any restriction throughout the day. In contrast, the control diet is only slightly more than the individuals' daily food requirement measured under plentiful food conditions (note that food consumption was measured on an *ad libitum* diet). Therefore, the control diet (i.e., 110% of ADFI) can still be considered as limited dietary regime if an individual's daily energy requirement is increased (e.g. for strategic fattening or regaining mass; Gosler, 1996). We used a randomized block design during the experiment, with each block containing one food-restricted and one control individual. We tested four blocks (i.e., 8 birds) per day, to minimize the bleeding time. Each treatment lasted for three consecutive days (trial; **Figure 1**).

The order of treatments was randomized so that 12 birds received the control and 12 birds the food-restricted treatment during the first trial. After the first trial, each individual received the opposite treatment for three consecutive days (trial 2). After two trials, all birds received the resting diet (*ad libitum* food enriched with mealworms) for 3 days before trials 3 and 4, for which we reversed the order of the treatments for each individual to separate the effect of the treatment sequence from a group effect. At the end of each trial, we measured the body mass, and took a blood sample (into a heparinized capillary tube) for hormone analyses. We did not sample the birds at the end of the resting period to minimize the number of blood samplings while focusing on the hormonal responses to the restricted vs. the control diet. We refer to the first part of the experiment (trials 1-2) as "week 1" and the second part of the experiment (trials 3-4) as "week 2" (**Figure 1**).

On each sampling day, we randomized the sampling order for individuals. Blood sampling was always carried out by two experimenters and two assistants. The assistants caught the particular birds following the previously randomized sampling order and gave them to the experimenters, who were blind to the treatment. The assistants also recorded the bleeding time and morphometric variables measured by the experimenters. Bleeding was carried out by puncturing the brachial vein with a 26G sterile needle. We took a maximum of 140 μl blood from each individual, which corresponds with the international ethical standards in blood sampling (Owen, 2010). The bleeding of the first birds always started at 09:00. We recorded when the first person entered the housing facilities and when the blood sample was collected (bleeding time average: 5 min 36 s [min: 1 min 7 s, max: 11 min 48 s]). Preliminary analyses showed that IGF-1 levels were not related to bleeding time, and adding bleeding time never improved the model fit, therefore, this variable was discarded from subsequent analyses. All birds were fed immediately after blood sampling. Blood was kept on ice and centrifuged within one hour to separate plasma from erythrocytes. We stored the samples at −20°C until further processing.

To minimize observer bias, all measurements (during the experiment and hormone assays) were performed blindly for the individuals' treatment.

IGF-1 Assay

Plasma IGF-1 levels were measured in a competitive ELISA developed in our laboratory at the University of Debrecen, which was validated for Bearded reedlings and described previously in Mahr et al. (2020). Briefly, 96-well microplates were coated overnight at 4°C with 100 μl of an antibody raised against IGF-1 in rabbits. The capture antibody was incubated for 2 h at room temperature (24°C) with 20 μl known concentrations of synthetic chicken IGF-1 in serial dilutions starting at 500 ng/ml or 20 μl of sample and 100 μl biotinylated IGF-1. After incubation,

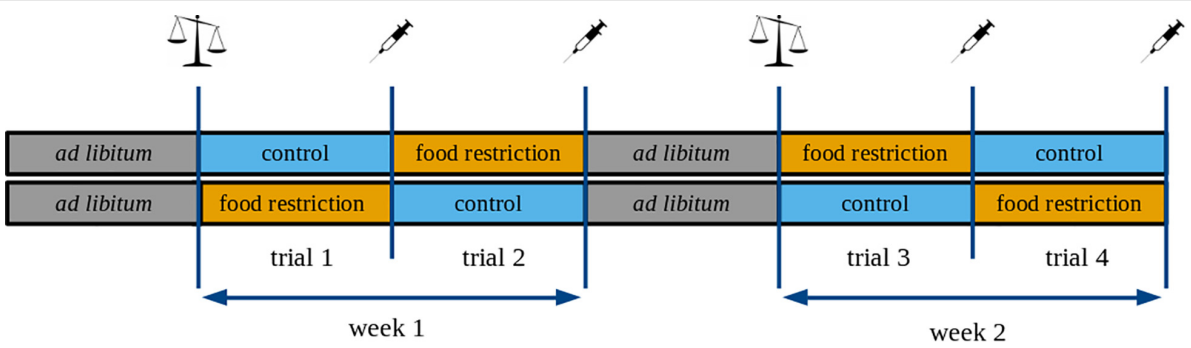

FIGURE 1 | Schematic illustration of the experimental design. Every treatment lasted for three consecutive days, and we called these periods "trials". Every bird received the control and the food-restricted treatment twice. We refer to the first replicate as "week 1" and to the second replicate as "week 2". The syringe icon denotes blood sampling and body mass measurements, whereas the balance icon indicates only body mass measurements.

the microplate was washed three times with 250 μl of PBS buffer containing 0.025% Tween 20, and 100 μl of streptavidin-horseradish peroxidase conjugate was added to all wells and incubated at room temperature for 30 min. After washing, 100 μl of tetra-methyl-benzidine was added to the wells and incubated at room temperature for 30 min. The enzymatic reaction was stopped by adding 100 μl of 1 M H_2SO_4, and optical density (OD) was measured at 450 nm (reference at 620 nm) using a Tecan F50 microplate reader. We used chicken plasma in quadruplicates to determine intra- and inter-assay coefficient of variation (6.8 and 10.88%, respectively). We were not able to calculate the concentration from three samples of IGF-1 because of insufficient plasma volume.

Statistical Analysis

We analyzed our data in a Bayesian framework, using R version 3.6.2 (R core team, 2019) and the package "MCMCglmm" (Hadfield, 2010). First, we analyzed how the treatment affected the body mass of the birds. We fitted a trivariate mixed-effects model, where body mass measured after each treatment period (*ad libitum*, control, or food-restricted) was used as response variable. The experimental week and the order of treatments (food restriction followed by control "RC" or control followed by food restriction "CR") and sex were used as fixed effects. Random intercepts were included for individual identity. This trivariate model allowed us to estimate the individual variance in body mass for *ad libitum*, control, and food-restricted diets separately, and the covariance between these terms. Based on these values, we calculated the among-individual cross-context (cross-treatment) correlations, which indicate intra-individual variation in the response given to the treatment (Dingemanse and Dochtermann, 2013; Houslay and Wilson, 2017). A high cross-context correlation (r close to 1) indicates consistent differences between individuals, e.g., a heavy bird under an *ad libitum* diet is likely to remain the heaviest (albeit with lower body mass) in the control and food-restricted diet as well. However, a lower r-value indicates that individuals react to different conditions differently (i.e., reaction norms cross over).

Second, we analyzed how the treatment affected IGF-1 concentrations. We used a univariate mixed-effects model with IGF-1 as the response variable, individual identity as the random intercept, and experimental week, treatment order group, treatment, and body mass as fixed effects. However, because body mass varies both among- and within-individuals, in this model, we partitioned the variance explained by body mass into among-individual and within-individual components (van de Pol and Wright, 2009). Among-individual body mass specifies the individual-specific average body mass, which explains variance due to consistent differences among individuals (e.g., a generally heavy vs. a light bird). On the other hand, the within-individual body mass component expresses the changes in body mass due to the specific dietary treatments.

Finally, we analyzed the covariation between IGF-1 and body mass. To do so, we built a multivariate model containing standardized body mass and IGF-1 (i.e., zero-centered and divided by the standard deviation), each included as a separate variable at each treatment level. Because blood samples were only collected after food-restricted and control (but not *ad libitum*) diets, this model contained four response variables (food-restricted and control treatment levels for both body mass and IGF-1). Individual identity was included as a random intercept. Experimental week, treatment order group, and sex were added as fixed factors. As above, in the trivariate model for body mass, this model also assumed a multivariate normal distribution full variance-covariance matrix that estimated all specific level variances and covariances. We also built alternative models with identical random and fixed structures, where specific parts of the variance-covariance matrix were constrained to zero. The model fit of these alternative models was compared using the Deviance Information Criterion, the Bayesian alternative of the Akaike Information Criterion. In this multivariate model, we also calculated the conditional among-individual variance for treatment-induced IGF-1 levels as variance in IGF-1 levels minus the square of the body mass∼IGF-1 covariance divided by the variance in body mass, for each food treatment level. This value represents the among-individual variance in IGF-1 that is not accounted for by variation in body mass due to the food restriction (Dingemanse and Dochtermann, 2013). When credible intervals of the estimate do not overlap zero, it can be interpreted as a "significant" variation among individuals. To facilitate interpretation, we also provide Bayesian p-values for fixed effects (Hadfield, 2010). As for the body mass model, we also estimated the cross-context correlation for the IGF-1 response, which indicates whether individual reaction norms cross over (if lower than 1).

RESULTS

Body Mass

Males were heavier than females, but sex did not interact with any other variables. The dietary treatment affected body mass in a complex manner, where body mass differed between treatments, the week, the order of treatments and the interaction between order and treatment (**Table 1**). Experimental food restriction had a strong effect: body mass declined in all individuals (**Figure 2** and **Supplementary Figure 1**). However, this body mass loss during food restriction was stronger when it followed the *ad libitum* diet than when it followed the control diet. This difference in the severity of body mass loss depending on the food availability in the preceding period was similar in both groups that differed in the order they received the treatments (**Figure 2** and **Supplementary Figure 1**). The dietary conditions that the birds experienced in the preceding period also affected body mass change during the control treatment. Despite having access to 110% of their daily food requirement, birds lost body mass during control treatment if it followed the *ad libitum* diet. However, if the control treatment followed the restricted (70%) diet, birds regained mass during this period (**Supplementary Figure 1**). These effects resulted in a pattern that when birds received the control treatment after *ad libitum* (order "CR"), then body mass after control treatment was mid-way between *ad libitum* and restricted diet. However, when the control treatment followed the food restriction (order "RC"), the difference between body mass measured after control and restricted diet was more pronounced (**Figure 2**). Also,

TABLE 1 | The trivariate mixed-effects model showed that the experimental food restriction strongly affected the birds' body mass.

	estimate	95% CI (lower, upper)	pMCMC
Mass (AL)	0.215	−0.247, 0.662	0.340
Mass (C)	−0.355	−0.791, 0.073	0.109
Mass (FR)	−1.115	−1.533, −0.698	<0.001
Sex (M)	0.509	0.030, 0.982	0.037
Week	0.456	0.193, 0.721	0.002
Group (RC)	−0.170	−0.440, 0.100	0.206
Mass (C) × Week	−0.108	−0.420, 0.192	0.482
Mass (FR) × Week	−0.004	−0.283, 0.285	0.964
Mass (C) × Group (RC)	0.329	0.019, 0.649	0.043
Mass (FR) × Group (RC)	−0.350	−0.645, −0.073	0.020

All birds lost weight due to food restriction. Furthermore, body mass loss was stronger if it happened after an ad libitum period. Mass (AL) is the body mass during ad libitum period, Mass (C) is the body mass during control diet, Mass (FR) is the body mass during the restricted diet. Group (RC) is when control diet followed the restricted diet and Group (CR) is the opposite. Body mass was standardized (mean = 0, SD = 1) before the analyses. CI denotes 95% Bayesian credible intervals, pMCMC denotes the Bayesian p-value.

during the mid-experiment recovery (*ad libitum*) phase, birds increased their body mass so much that they became heavier by the beginning of week 2 compared to the beginning of the experiment (week 1). The latter effect was especially pronounced in the group that finished week 1 with the restricted diet (**Supplementary Table 1**; **Figure 2**; **Supplementary Figure 1**). These results were corroborated by univariate Bayesian analyses (**Supplementary Table 1**).

While individuals differed in their average body mass, the change in body mass was mostly parallel across feeding regimes among individuals (**Supplementary Figure 2**). Individual correlation of body mass across treatments was very high (*ad libitum* – control: 0.95, control - food-restricted: 0.94), indicating that consistent differences in body mass between the individuals remained unchanged despite the experimentally induced changes in body mass (**Supplementary Figure 2**).

IGF-1

Univariate analyses of IGF-1 showed that IGF-1 levels were higher during the food-restricted diet than during the control (4.40 [0.39; 8.22], $p = 0.02$) once we controlled for variation in body mass (**Figure 3**). To investigate this effect further, we divided body mass into two variance components: among-individual body mass (reflecting average mass differences among birds) and within-individual changes in body mass (reflecting experimentally induced loss and regain of body mass). This model showed that while among-individual body mass was positively related to IGF-1 (4.95 [0.75; 9.01], $p = 0.02$), within-individual changes in body mass tended to be in the opposite direction: i.e., body mass loss was associated with an increase in IGF-1 levels (resulting in a negative slope: −29.85 [−63.21; 2.05], $p = 0.06$), and this effect was the strongest in birds with low average body mass (1.81 [−0.27; 3.80], $p = 0.07$, **Figure 4**). However, the effects of within- and among individual body mass on IGF-1 had wide credible intervals that slightly encompassed 0 and were considered statistically marginally non-significant.

Males tended to have higher IGF-1 levels than females (5.92 [−0.25; 12.15], $p = 0.062$). Since males were also heavier than females, we repeated the above model by including sex as an additional variable. Controlling for sex did not improve model fit (ΔDIC = −0.2), and sex did not explain significant variation in IGF-1 levels (4.17 [−2.6; 10.57], $p = 0.182$). At the same time, the other estimates remained similar, indicating that the effects of sex were driven mainly by the higher body mass of males. This was further supported by a model showing that individuals heavier than the median body mass of their sex were more likely to decrease their IGF-1 levels in response to the treatment (−6.87 [−12.12; −1.95], $p = 0.009$), whereas individuals lighter than the average showed the opposite pattern and were more likely to increase their IGF-1 levels (5.09 [1.66; 8.52], $p = 0.003$), (**Figure 4**).

When body mass and IGF-1 were analyzed together in a multivariate model (**Table 2**), we found significant positive covariance between body mass and IGF-1 under the control diet, corroborating results from the univariate analysis that birds with higher average body mass also had higher IGF-1 levels. The model that estimated covariance between body mass and IGF-1 had better support than the one where body mass was not allowed to covary with IGF-1 levels (ΔDIC = 5.2). IGF-1 levels after food restriction tended to be related to treatment-induced body mass, although this effect remained marginally non-significant (0.19 [−0.06; 0.49]). Furthermore, we found that individuals differed significantly in their IGF-1 levels in response to the food restriction. Still, this difference in individual IGF-1 response was independent of the body mass change caused by food restriction (conditional among-individual variance = 0.36 [0.01; 0.94], repeatability = 0.44). The analysis of change in IGF-1 levels from control to food restriction revealed significant individual variation and crossing reaction norms (**Supplementary Figure 3**), indicating that some individuals decreased, whereas others increased IGF-1 in response to the treatment (cross-context correlation: $r = 0.70$, this model is considerably better supported than the model, where the cross-context correlation was fixed to 1: ΔDIC = 19.6).

DISCUSSION

Our experiment revealed that in response to food restriction, bearded reedlings showed marked individual differences in their IGF-1 reaction norms. While, as predicted, in some individuals, IGF-1 levels decreased in response to a restricted diet, the majority of the birds showed no response or even an increase. We also showed that heavier individuals had higher overall IGF-1 levels, and were more likely to decrease IGF-1 in response to the food-restriction. These results uncover the presence of an individual by environment interaction (I × E) and may have important implications for the evolution of IGF-1-related hormonal phenotypes in this species.

All birds lost weight during the restricted dietary regime, demonstrating that the experimental treatment was sufficient to simulate low food availability. Furthermore, during the mid-experiment *ad libitum* diet, birds increased their body mass to a higher level than their initial body mass at the beginning of

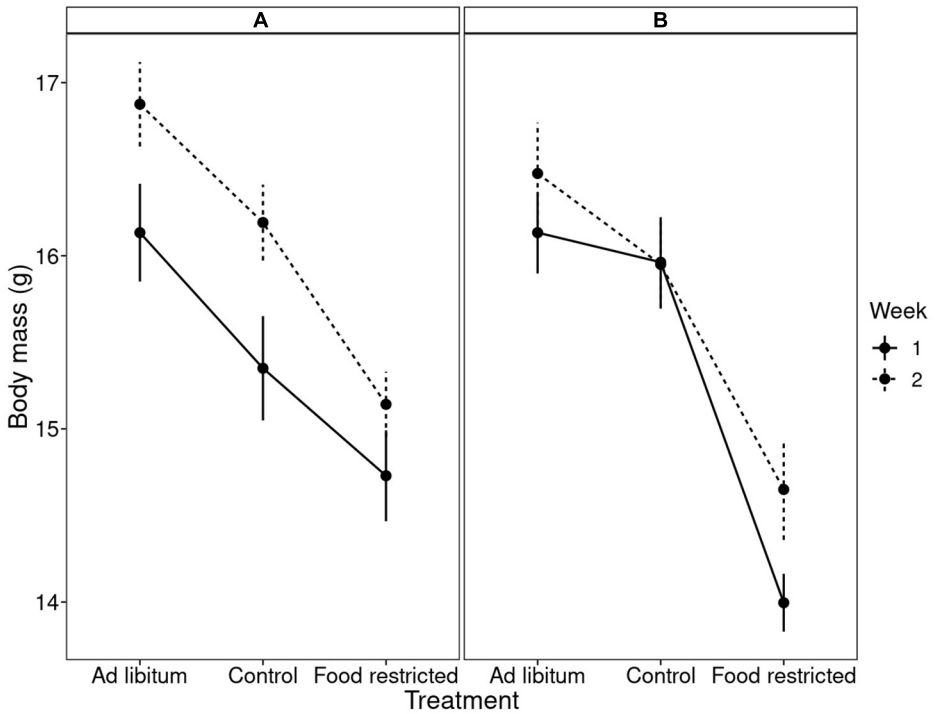

FIGURE 2 | Mean ± SE body mass in bearded reedlings after different dietary treatments. The *x*-axis shows the type of dietary treatments while the two panels show in which order the birds received these treatments (see also **Supplementary Figure 1**). **(A)** Indicates control diet followed by restricted diet, and **(B)** means restricted diet followed by control diet. Solid and dashed lines indicate week 1 and 2 of the experiment, respectively. Note that body mass variation was affected by treatment, week, the order of treatments and the treatment × order interaction.

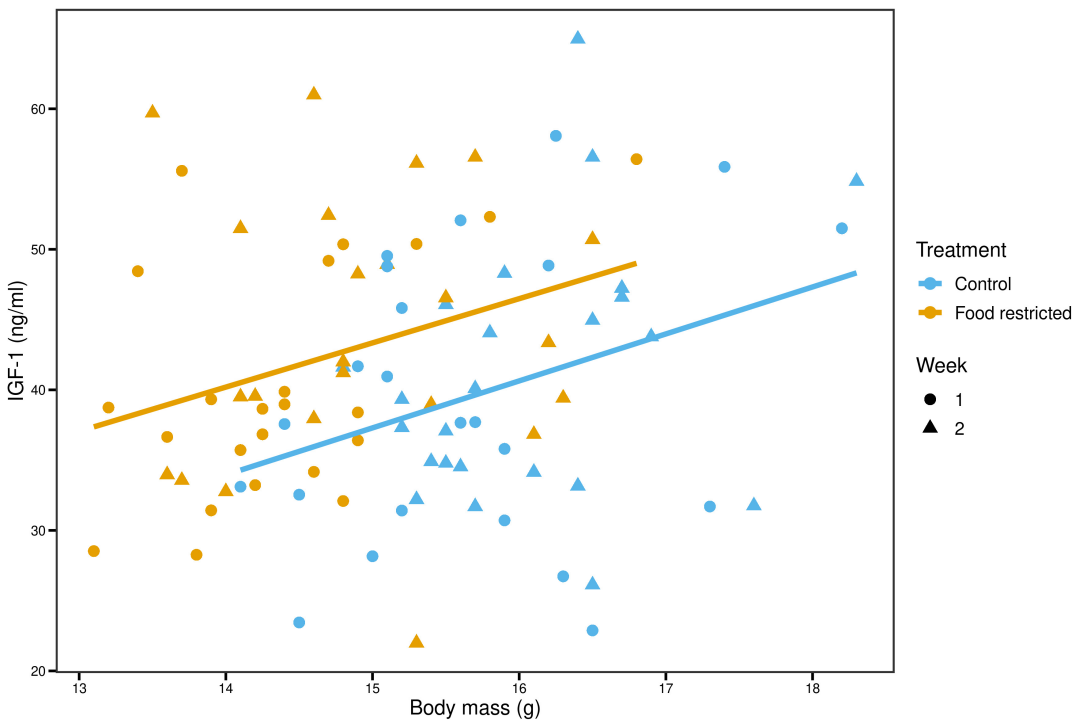

FIGURE 3 | Birds with higher average body mass have higher IGF-1 levels. Blue and orange solid lines show the among-individual relationships for control and food-restricted treatment, respectively.

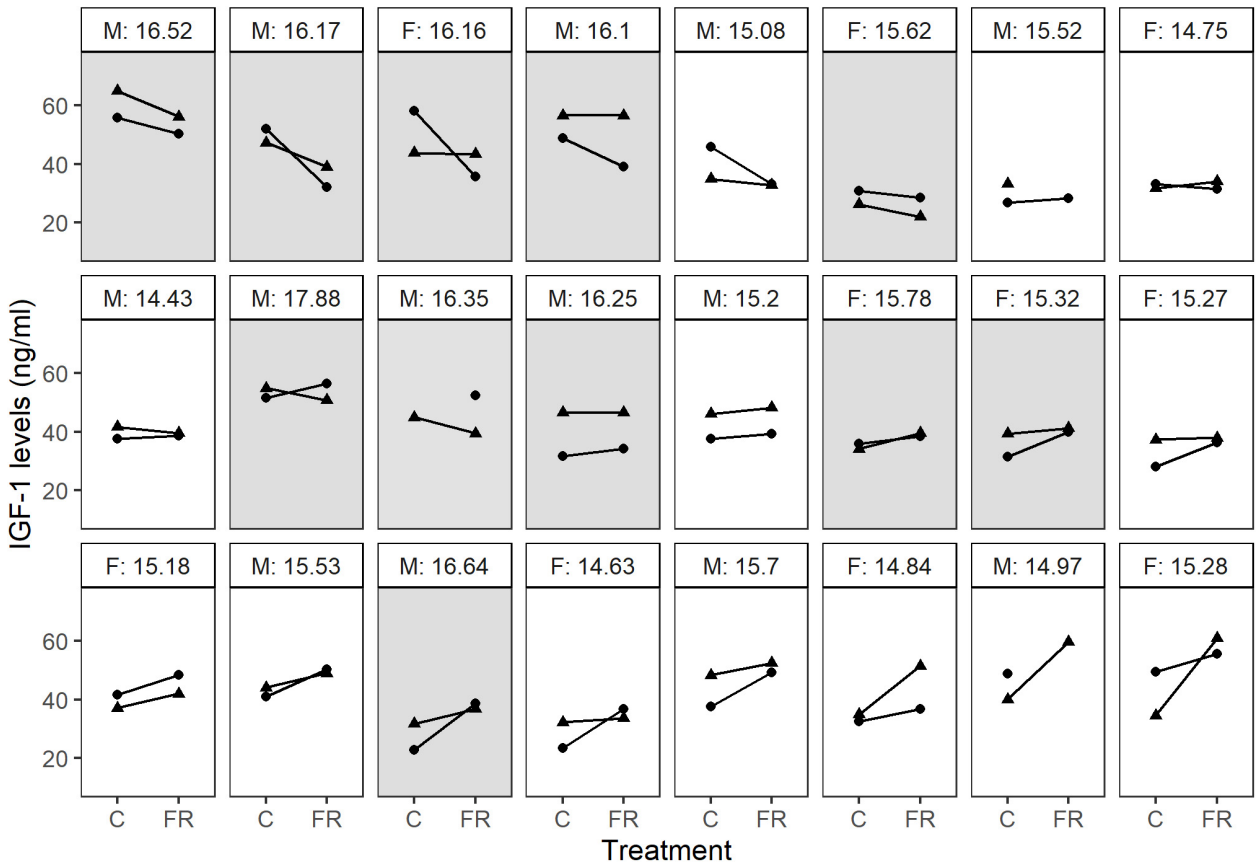

FIGURE 4 | IGF-1 reaction norms in response to food restriction show large among-individual variation. Each panel represents an individual; dots or triangles connected by a line denote IGF-1 levels measured after control (C) or food-restricted (FR) treatment during the first and the second half of the experiment (week 1: dots, week 2: triangles). The text above the panel show the sex (M: males, F: females) and the average body mass (g) for each individual. Light and dark panels indicate individuals lighter and heavier than the median mass of their sex, respectively. For three individuals, one of the samples are missing.

the experiment. Finally, the decrease in body mass was stronger when the restricted diet followed the *ad libitum* feeding regime than when it followed the control regime (**Figure 2**). Our findings are in line with the adaptive regulation hypothesis, namely that individuals should maintain their current energetic status during low food availability and regain reserves during periods of high food availability to reduce the future risk of starvation (Witter et al., 1995; Fauchald et al., 2004).

The results on body mass resemble findings on House sparrows by Lendvai et al. (2014), even though the duration of the food-restriction period was shorter (3 days vs. 1 week) and the severity of the food restriction was lower in the current study (30 vs. 40% restriction). Our study design allowed us to separate the effects of the treatment sequence from a group effect because, at the midpoint of the experiment, the order of treatments was reversed in each experimental group (**Figure 1**). These results emphasize that individual responses to a standardized food restriction depend on previously experienced resource availability (Acquarone et al., 2002; Cucco et al., 2002; Fokidis et al., 2012). During the transition from *ad libitum* to control diet, birds were presented with less food in their feeder. Although they could still meet their daily energetic requirements (the control diet consisted of 110% of their individual daily food intake, measured under conditions when food was available in plentiful conditions), the lower amount of food left in the feeder at the end of the day may have been a visual cue for anticipating a deterioration of nutritional conditions. On the contrary, the 110% of daily food requirement may be seen as a significant improvement for birds who switched from the restricted treatment. As a consequence, while birds entering the control treatment from *ad libitum* conditions lost body mass during the control regime, birds previously experiencing food restriction regained body mass during the same treatment (**Supplementary Figure 1**). These responses indicate that birds may anticipate future resource availability based on previous experiences.

The perception of potential shortages in food resources might also determine individual physiological responses that allow individuals to mitigate the costs of low nutrient availability if they anticipate a decrease in nutritional conditions. While these dynamic changes in body mass were strong and consistent among individuals (**Supplementary Figure 2**), we found that changes in IGF-1 levels were markedly different among individuals (**Figure 4** and **Supplementary Figure 3**). First, IGF-1 levels were repeatable within individuals and positively related to the average body mass, i.e., heavier birds had higher IGF-1 levels. Males were heavier than females and tended to have higher IGF-1 levels [as shown before in Tóth et al. (2018)], but once we controlled for body mass, the sex difference in hormone levels disappeared. Our

TABLE 2 | Variance-covariance matrix from the multivariate MCMCglmm model including IGF-1 and body mass under each treatment (control, "C" or food-restricted, "FR").

	IGF1 (C)	IGF1 (FR)	Mass (C)	Mass (FR)
IGF1 (Control)	0.72 [0.13; – 1.33]			
IGF1 (FR)	**0.40 [0.04; – 0.86]**	0.62 [0.04; – 1.19]		
Mass (Control)	**0.33 [0.03; – 0.71]**	0.17 [–0.11; – 0.52]	0.46 [0.09; – 0.86]	
Mass (FR)	**0.32 [0.05; – 0.67]**	0.19 [–0.06; – 0.49]	**0.29 [0.04; – 0.55]**	0.32 [0.06; – 0.61]

The diagonal shows the variances, while off-diagonal elements correspond to the covariance between the variables. Significant covariance terms are highlighted in bold font.

findings on the positive relationship between body mass and IGF-1 are consistent with the available (albeit scarce) literature on fish (Cameron et al., 2007), reptiles (Crain et al., 1995; Sparkman et al., 2009), and mammals (Lewin et al., 2016; Tighe et al., 2016), although our result alone does not imply a causal relationship.

Second, individuals differed in their hormonal response to food restriction. While some individuals decreased their IGF-1 levels when food became scarce, others showed little response or even increased their IGF-1 levels (**Figure 4**). This difference remained after controlling for individual variation in body mass changes. Intriguingly, the variation in reaction norms was associated with the average body mass: relatively lighter birds were more likely to increase IGF-1 levels, while heavier birds were more likely to show a reduction in IGF-1. The restricted dietary regime is expected to decrease IGF-1 expression and secretion across invertebrates and vertebrates (Morishita et al., 1993; Schew et al., 1996). In contrast with our predictions and previous findings, we did not observe a consistent decrease in IGF-1 levels during food restriction, but we found significant among-individual variation (I × E) in the IGF-1 response to food restriction. Therefore, the question arises why food restriction had diverse effects on IGF-1 levels in adult bearded reedlings and whether this physiological response might facilitate survival under conditions of low food availability and constitute a possible coping mechanism to unpredictable environmental cues?

IGF-1 strongly affects energy metabolism including the elevation of glucose uptake without lowering free fatty acid levels (Kastin, 2013; Aguirre et al., 2016). It also promotes the formation of fat reserves via regulation of preadipocyte differentiation and increased lipogenesis (Smith et al., 1988; Scavo et al., 2004), which allows organisms to preserve energy to survive harsh environmental conditions. However, after preadipocytes differentiate, they stop expressing IGF-1 receptors. Therefore, in adipose tissues, only a high concentration of IGF-1 can effectively prevent lipolysis, and stimulate glucose transport (DiGirolamo et al., 1986). Based on our study and the role of IGF-1 in anabolic processes, we suggest that individuals might express different strategies when confronted with reduced food resources based on their initial energy status. Lighter (i.e., lean) individuals might produce more IGF-1 to maintain their energy homeostasis and mitigate the adverse effects of apoptosis and protein degradation, such as muscle atrophy (Musarò et al., 1999; Timmer et al., 2018). Furthermore, increasing IGF-1 during moderate or early stages of fasting might prevent protein degradation and facilitate the maintenance of cell growth and proliferation until energy becomes available (Scavo et al., 2004). On the contrary, heavier (i.e., fat) birds with decreased IGF-1 levels may be able to suppress insulin activity and increase blood glucose levels via gluconeogenesis, which is enough to maintain their normal life processes during harsh conditions and favor survival (Yakar et al., 2004). This hypothesis remains to be tested.

Only a few studies have suggested that food restriction may increase IGF-1. For example, Ayson et al. (2007) observed in rabbitfish that the hepatic IGF-1 mRNA level was higher in the starved (no food for 15 days) group than in the control group, albeit only in the early part of starvation (2^{nd} and 3^{rd} days). During prolonged starvation (15^{th} –18^{th} days), the IGF-1 mRNA level became significantly lower in the starved group compared with the controls. Ayson et al. (2007) suggested that previous studies may have missed the early increase of IGF-1 in response to starvation. However, another study on broiler chicken found that food restriction resulted in higher IGF-1 levels than controls throughout the study (from 15 to 28 weeks of age) (Hocking et al., 2007). We also found that female canaries responded to food restriction by increasing IGF-1 levels during breeding (Hargitai et al., 2022). These studies only analyzed the overall response to food-restriction, but here we show that within a single population, individuals may differ markedly in their physiological response to changes in nutritional conditions.

IGF-1 can also interact with other physiological parameters to modulate phenotypic responses. For example, Lodjak et al. (2016) showed in pied flycatcher nestlings that IGF-1 and glucocorticoid levels are positively related under high food abundance, while this relationship turns negative under low food abundance. They hypothesized the existence of a threshold level in physiological conditions over which the relationship between the two hormones can change. Our finding that heavier birds were more likely to decrease IGF-1 levels under food-restriction, whereas light birds were more likely to increase it may support the existence of such a physiological turning point, which may explain the variance of IGF-1 reaction norm during food-restriction. Accordingly, the relationships between IGF-1 and other physiological traits such as glucose levels, glucocorticoids and a marker of oxidative stress can be reorganized during environmental challenges (Vágási et al., 2020). These results suggest that the relationship between IGF-1 and other physiological factors is context- and condition-dependent and their joint effect on life-history or fitness-related traits is still unexplored in natural populations.

It should also be considered that with few exceptions, most of our knowledge about the effects of food availability on circulating IGF-1 levels comes from experiments conducted on model organisms (e.g., mice) in controlled laboratory settings and/or farm or breeding facilities (Puig and Tjian, 2006; Dantzer and Swanson, 2012). The feeding patterns of these animals (e.g., maintained unlimited access to food in lab condition) may not reflect the natural feeding habits of wild populations. Furthermore, laboratory and farm animals have been artificially selected on specific traits, such as rapid growth and an early onset of the reproduction, and therefore display different life

histories and physiological phenotypes (e.g., metabolism) from wild animals (Petersson et al., 1996; Leili et al., 1997; Geiser et al., 2007; Auer et al., 2016; Bolstad et al., 2017). Considering that these traits correlate positively with IGF-1 levels (Frystyk et al., 1999), the measured physiological responses to low food availability may not reflect those of natural populations. Even though we conducted our experiments in captive animals, our study animals came from a natural population, therefore, it can represent the natural variation in IGF-1 response to food restriction.

Here, we uncovered large individual variation in the IGF-1 reaction norms, and the next step is to identify how these phenotypic differences are related to fitness. Dietary restriction is the most robust intervention that extends lifespan and delays ageing in various organisms, and it has been suggested that adaptive plasticity in the insulin/insulin-like signaling pathway underpins the physiological basis of this effect (Regan et al., 2020). Indeed, variation in IGF-1 levels has been connected to various life-history traits in vertebrates (Dantzer and Swanson, 2012). While IGF-1 is most often studied during post-natal growth, there is growing evidence that variation in IGF-1 is connected to fitness-related traits, such as lifespan and fecundity (Lodjak and Mägi, 2017), oxidative stress (Vágási et al., 2020) and ornament expression (Mahr et al., 2020; Lendvai et al., 2021) in adult birds. Given these relationships and that after an initial challenge IGF-1 levels do not return immediately to the baseline level (Gabillard et al., 2006), we expect that the way individuals react to variation in food availability may have longer-term, adaptive consequences. Therefore, the temporary lack of food could have a prolonged effect on reproduction via condition dependent IGF-1 regulation.

In conclusion, our study provides novel information on the existence of repeatable individual variation and multiple reaction norms in IGF-1 levels in response to food restriction. The variability of reaction norms can contribute to maintaining the genetic diversity within populations. This has a major ecological and evolutionary consequence because high genetic diversity reduces bottlenecks caused by environmental challenges and also involves the possibility of fast adaptation to the new circumstances (Fisher, 1930; Bouzat, 2010). IGF-1 showed a highly plastic response to one of the major environmental challenges (food deprivation). Therefore, we propose that IGF-1 might hold a prominent role in shaping adaptive responses to environmental changes, among other physiological variables, which remains to be tested.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Környezetvédelmi és Természetvédelmi Főosztály, Hajdú-Bihar Megyei Kormányhivatal.

AUTHOR CONTRIBUTIONS

ZT and ÁZL designed the experimental protocol and conducted the statistical analyses. ZT, GÖ, LŐ, and ÁZL performed the experimental procedures with contribution from KM. ZT and ÁZL performed the lab analyses. ZT, KM, and ÁZL wrote the manuscript with contributions from GÖ and LŐ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.826968/full#supplementary-material

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Ontogeny and individual heterogeneity of the corticosterone stress response in a wild altricial seabird, the snow petrel (*Pagodroma nivea*)

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In the current context of global change, there is evidence of a large inter-individual variability in the way animals physiologically respond to anthropogenic changes. In that context, the Hypothalamus-Pituitary-Adrenal (HPA) axis and the corticosterone stress response are of primary importance because they are thought to govern the ability of individuals to adjust to stress. Several studies have reported that this stress response is variable among adults and they have successfully linked this variability with abiotic and biotic factors. However, the inter-individual variability of the glucocorticoid stress response has rarely been examined during the developmental phase in wild vertebrates, and its potential ecological determinants remain unclear. In this study, we examined the ontogeny of the corticosterone stress response in an altricial seabird species (i.e., how corticosterone levels increase in response to a standardized stress protocol), the Snow petrel. We reported a strong heterogeneity of the corticosterone stress response at all ages in snow petrel chicks (11-, 20-, and 37-days old chicks). Although the magnitude of this corticosterone stress response decreases with the age of the chick, we also found that this corticosterone stress response was repeatable throughout the post-hatching developmental period (repeatability: $r > 0.50$ for stress-induced corticosterone levels after a 30-min restraint). Importantly, this glucocorticoid stress response was negatively associated with the body condition of the chicks (i.e., mass corrected for body size), and previous exposure to sampling was associated with a dampened corticosterone stress response. However, we did not find any link between parental traits (parental condition or parental corticosterone stress response), nest quality, hatching

date, and the chick's corticosterone stress response. Our study suggests that the corticosterone stress response is a consistent individual trait that is affected to some extent by post-natal conditions, and which differs among individuals very early in life.

KEYWORDS

stress, glucocorticoids, development, repeatability, HPA axis

Introduction

In the current context of global change, there is an urgent need to appropriately assess to what extent wild populations of vertebrates can persist when confronted to anthropogenic changes (Newbold, 2018; Warren et al., 2018). To achieve this goal, it is acknowledged that individual heterogeneity is of primary importance because different individuals may differ in the way they react to change and in the way they sustain the costs of environmental changes (Clutton-Brock and Sheldon, 2010; Wilson and Nussey, 2010; Cam et al., 2016; Vedder and Bouwhuis, 2018; Forsythe et al., 2021). Indeed, there is strong evidence that there is a large inter-individual variability in the way individuals respond to stress with important fitness consequences. In that context, the concept of allostasis is crucial to consider because it is involved in the ability of individuals to achieve stability through change (McEwen and Wingfield, 2003). Allostasis-related mechanisms allow the individual to adjust to environmental changes and they involve several organismal systems (Wingfield, 2003; Romero et al., 2009). Among those, the Hypothalamus-Pituitary-Adrenal (HPA) axis is commonly studied because it is classically involved in the so-called "stress response" (Wingfield et al., 1998; Wingfield, 2003), and it is thought to play a role in the ability of vertebrates to cope with global change (Angelier and Wingfield, 2013). In response to stressors or environmental constraints, this endocrine axis is activated and it results in the quick release of significant amounts of glucocorticoids in the bloodstream (Sapolsky et al., 2000). In turn, glucocorticoids–corticosterone in birds, amphibians, reptiles and rodents–mediate behavioral and physiological responses that aim to help the organism survive the stressor (Landys et al., 2006). The inter-individual variability of this corticosterone stress response has been extensively studied in birds (Cockrem, 2007, 2013a,b) and its role in mediating inter-species and inter-individual in key life-history decisions has been convincingly demonstrated (Wingfield and Sapolsky, 2003; Bokony et al., 2009; Hau et al., 2010). However, the origin of this inter-individual variability remains relatively overlooked despite its importance to understand to what extent individuals can adjust to environmental changes (Angelier and Wingfield, 2013; Romero and Wingfield, 2016).

Importantly, the corticosterone stress response seems repeatable within individuals during the adult life (Taff et al., 2018) and this suggests that the corticosterone stress response is an individual trait with a somewhat limited flexibility during adulthood (Rensel and Schoech, 2011). Accordingly, a few studies on wild birds have shown that genetic components could be important drivers of inter-individual differences in the corticosterone stress response (Satterlee and Johnson, 1988; Evans et al., 2006; Almasi et al., 2010; Angelier et al., 2011; Baugh et al., 2012; Jenkins et al., 2014; Béziers et al., 2019). Recent studies have clearly demonstrated that the developmental period is also crucial to consider when explaining inter-individual differences in the corticosterone stress response because it is a key stage for its ontogeny (Wada et al., 2009; Schoech et al., 2011). Indeed, developmental conditions can have significant effects on the HPA axis and the resulting corticosterone stress response that can persist until adulthood [e.g., Marasco et al. (2012) in the Japanese quail]. For example, developmental nutritional restrictions have been shown to alter the functioning of the HPA axis later in life (e.g., Jimeno et al., 2018), and experimental exposure to corticosterone levels during the developmental phase has also been shown to affect the magnitude of the corticosterone stress response later in life (e.g., Crino et al., 2014; Grace et al., 2020).

Despite these significant advances, most of these studies have focused either on the adult corticosterone stress response only or on a single measure of the response during the developmental phase, limiting our ability to understand individual trajectories in the ontogeny of the corticosterone stress response. Therefore, it remains unclear how the magnitude of the corticosterone stress response changes within individuals over the developmental phase, and what ecological factors could drive such changes. It remains also unclear whether the corticosterone stress response is repeatable within individuals during the developmental phase. In altricial chicks, a few studies have demonstrated that the corticosterone stress response is reduced or even suppressed in young chicks relative to juveniles or adults in altricial species (e.g., Sims and Holberton, 2000; Love et al., 2003; Wada et al., 2007;

Rensel et al., 2010; Bebus et al., 2020; Jones et al., 2021) and this stress hypo-responsive period [SHRP, stress hyporesponsive period, *sensu* Wada (2008)] at an early age has also been demonstrated in an altricial laboratory bird model, the zebra finch (Wada et al., 2009). Opposite results were found in captive precocial species with an effective stress response at a very early age (e.g., Ericsson and Jensen, 2016) and it has been linked with the relative independence of precocial birds at young ages (Wada, 2008). Interestingly, the same pattern was found in two altricial bird species [gray faced petrels, Adams et al. (2008); green-rumped parrotlets, Berg et al. (2019)]: young chicks were able to mount a strong stress response—similar to the adult corticosterone stress response—almost immediately after hatching. Therefore, additional studies are necessary to better understand the ontogeny of the stress response in developing chicks of wild birds, and its ecological determinants.

In this study, we examined the ontogeny of the corticosterone stress response in an altricial seabird species, the Snow petrel (*Pagodroma nivea*) and we investigated the potential determinants of inter-individual variation in the corticosterone stress response in chicks. Snow petrel chicks were sampled three times to (1) investigate how the corticosterone stress response changes over the course of the post-hatching developmental period; (2) document the inter- and intra-individual variability of the corticosterone stress response; (3) determine the influence of abiotic and biotic factors on the ontogeny of the corticosterone stress response. According to previous studies conducted in altricial species [Wada et al., 2007, 2009; Rensel et al., 2010, but see Adams et al. (2008)], we predicted that the magnitude of the corticosterone stress response will increase over the course of the developmental period as the HPA system is developing (prediction 1). Because the corticosterone stress response is at least partly heritable (Evans et al., 2006; Béziers et al., 2019) and has been shown to be repeatable from the developmental period to adulthood (e.g., Wada et al., 2008), we also predicted that the corticosterone stress response will be correlated to that of the parents (prediction 2) although this link may be tenuous because of the modulation of the stress response by body condition and parental care in breeding snow petrels (Angelier et al., 2009, 2020). We also predicted that this corticosterone stress response will be consistent within individuals throughout the development (i.e., repeatable, prediction 3). Finally, we predicted that the corticosterone stress response will be modulated by the internal state of the chick [i.e., body mass corrected for body size, hereafter "body condition," Lynn et al. (2003, 2010) and Angelier et al. (2015), prediction 4], by the post-hatching environmental conditions (i.e., nest site, repeated exposure to stress, prediction 5), and by proxies of parental quality [i.e., hatching date, Sauser et al. (2021), and parental body condition, Barbraud and Chastel (1999); prediction 6].

Materials and methods

Study species

Snow petrels were studied on Ile des Pétrels, Pointe Géologie Archipelago, Terre Adélie (66°40'S, 140°01'E), Antarctica. Snow petrels are long-lived birds with high survival probability and low fecundity (Chastel et al., 1993). They lay a single egg and parents alternate incubating the egg until hatching. Incubation period lasts by average 45 days. After a period of brooding that is limited to approximately 9 days (Barbraud et al., 2000), the chick is left unattended by both parents, which forage simultaneously at sea to feed their chick. Males and females provide roughly similar amounts of parental care in this species, and by average, the chick receives a meal every 17 h (Barbraud et al., 1999) but the timing of the last meal was not known in our study. The chick developmental period lasts approximately 42 days (Barbraud et al., 2000) and the chicks then fledge and leave the colony until their first breeding attempt at approximately 10 years old (Chastel et al., 1993).

Hatching date, body condition, and the corticosterone stress response

In January 2012, 27 nests were monitored for hatching date by daily visual inspection of the nests. After hatching these nests were followed to monitor the growth of the chick and the ontogeny and the repeatability of the corticosterone stress response (prediction 1 & 3). In snow petrels, hatching date has been linked with parental age, and fledging success (Sauser et al., 2021), suggesting that hatching date is a reliable proxy of parental quality (prediction 6). After hatching, all nests were left undisturbed for 11 days and 15 chicks were measured and sampled when they were 11-days old, 20-days old and 37-days old. In addition, groups of seven and five chicks were randomly selected and left undisturbed until 20-days old and 37-days old (instead of sampling at 11-days old), respectively. This allowed us to test if the corticosterone stress response was affected by repeated captures/sampling (i.e., repeated exposure to stress, prediction 5) through habituation or sensitization. When nests were visited, the tarsus and the weight of each chick was measured with a caliper (±0.01 mm) and a spring scale (±5 gr), respectively. This allowed us to calculate the scaled mass index of each chick, as a proxy of body condition (Peig and Green, 2009), and to test its relationship with the corticosterone stress response (prediction 4). In addition, their corticosterone stress response was monitored by using a standardized stress protocol (Wingfield et al., 1992). Specifically, a first blood sample was collected from the chick within 3 min of capture to monitor baseline corticosterone levels. Then, the chick was restrained in a cloth bag for 30 min. A second and a third blood samples were collected after 15 and 30 min of restraint, respectively, to

monitor stress-induced corticosterone levels (hereafter, stress-induced corticosterone levels at 15 min and stress-induced corticosterone levels at 30 min). Four birds could not be sampled quickly enough to monitor baseline corticosterone levels at a given age (Romero and Reed, 2005).

Nest quality

Nest quality was assessed according to the structure of the nest to test the link between the chick's stress response and the quality of the nest (prediction 5). In snow petrels, the quality of the nest can be assessed according to the protection it offers to the chick against predators when the chick is left alone in the nest. In our study colony, South polar skuas (*Catharacta maccormicki*) often wander in the colony and opportunistically catch snow petrel chicks that are left alone in their nest. In that context, nest structure can play an important antipredator role by limiting the access to the chick by Antarctic skuas. In this study, we scored the nests that were monitored according to the protection it can offer to the chick against Antarctic skuas. A score of 1 referred to an open nest, which could be easily accessible to skuas. A score of 2 referred to a nest that is located between rocks but that remains partly accessible to skuas. A score of 3 referred to a nest that is located in a well-protected cavity and that is not accessible at all to skuas.

Parental corticosterone stress response and parental condition

During this field season (Austral summer 2011/2012), 35 breeding adult snow petrels were also captured during the incubation and the chick-rearing periods. All adults were captured while incubating their egg (N = 17) or brooding their chick (when the chicks were 5–20-days old, N = 18) at the nest. At capture, their tarsus and weight were measured with a caliper (±0.01 mm) and a spring scale (±5 gr), respectively. This allowed us to calculate the scaled mass index of each adult, as a proxy of body condition (Peig and Green, 2009) and to test the link between parental body condition and the chick's stress response (prediction 6). The SMI was computed for each i individual as follows: $SMI_i = M_i \times (L_o/L_i)^b$ where M_i and L_i are, respectively, the body mass and the tarsus length of the individual i, L_o, the arithmetic mean value of tarsus length for the whole study population and b the slope estimate of a standardized major axis regression of log-transformed body mass on log-transformed tarsus length [see Peig and Green (2009)]. In addition, the corticosterone stress response of the parents was monitored by using a standardized stress protocol (Wingfield et al., 1992), as previously described for the chicks. Thus, we were able to measure their baseline corticosterone levels (within 3 min of capture), and their stress-induced corticosterone levels (after a 30 min restraint). Note that the adults were not sampled after 15 min of restraint. Importantly, all 18 sampled chick-rearing adults were a parent of a sampled chick, allowing us to test whether the corticosterone stress response of a chick is related to the corticosterone stress response of one of its parents (prediction 2). Only one parent per nest was captured and sampled to reduce nest disturbance.

Laboratory analyses

Blood samples were centrifuged and plasma was separated from red blood cells. Plasma samples were then stored at−20°C until analyzed at the lab. Plasma concentrations of corticosterone were determined by radioimmunoassay, as described previously (Angelier et al., 2015). All samples were run in one assay and three reference plasma were run in duplicates to calculate the intra-assay CV (intra-assay CV: 7.07%).

Statistical analyses

All statistical analyses were performed with SAS statistical software (SAS University edition). Firstly, General Linear Mixed Models (GLMM, proc glimmix) were run to test the influence of the "time" factor (three levels, baseline levels, stress-induced levels after 15 min of restraint, stress induced levels after 30 min of restraint), the "age" factor (three levels, 11-days old chicks, 20-days old chicks, and 37-days old chicks), and their interaction on corticosterone levels of chicks. Because each chick was sampled several times, "chick identity" was added as a random factor to control for repeated measures. Secondly, and to test the difference in corticosterone levels between chicks and adults, GLMM were run to test the influence of the "time" factor (two levels, baseline, stress-induced levels after 30 min of restraint), and the status factor (five levels, 11-days old chicks, 20-days old chicks, 37-days old chicks, incubating adults, chick-rearing adults) on corticosterone levels. This second set of models was run to compare chicks and adults because stress-induced corticosterone levels after 15 min of restraint were available for the chicks only. Thirdly, GLMMs were run to test the impact of previous exposure to capture and handling stress on corticosterone levels of 20-days old chicks and 37-days old chicks. These GLMMs aimed to test the influence of the "time" factor (three levels, baseline levels, stress-induced levels after 15 min of restraint, stress induced levels after 30 min of restraint), the "age" factor (two levels, 20-days old chicks, and 37-days old chicks), body condition, the "previous capture" factor (two levels, previously captured or not) and their interaction on corticosterone levels of chicks., Body condition index was standardized per age to allow building such models (transformation to a Z-distribution). Hatching

date was also standardized (transformation to a Z-distribution). Fourthly, GLMMs were run to test the influence of the 'time' factor (three levels, baseline levels, stress-induced levels after 15 min of restraint, stress induced levels after 30 min of restraint), the "age" factor (three levels, 11-days old chick, 20-days old chicks, 37-days old chicks), body condition, "nest score," "hatching date," and their interactions on corticosterone levels of chicks. Because a specific data point had an important impact on the statistics (statistical outlier) and made some interactions almost significant (see **Table 1**), the same set of models was also run without this specific data point. Finally, GLMM were run to test the influence of the 'age' factor (three levels, 11-days old chicks, 20-days old chicks, and 37-days old chicks), parental baseline and stress-induced corticosterone levels, parental body condition, and their interaction on chick's corticosterone levels. Because each chick was sampled several times, "chick identity" was added as a random factor. Model selections were conducted using a step wise approach and by eliminating progressively non-significant terms (Burnham and Anderson, 2002). Model assumptions were checked by using diagnostic plots (SAS, residual panels and box plots option) to visualize the distribution and the variance of residuals. Repeatability analyses (rptR package, R software) were run to assess the repeatability of (1) baseline corticosterone levels, (2) stress-induced corticosterone levels after 15 min of restraint, (3) stress-induced corticosterone levels after 30 min of restraint within individuals. Chick identity and the "age" factor were included as random and fixed factors, respectively.

TABLE 1 Model selections to test the influence of the age of the chick (11-days old, 20-days old, 37-days old), time of sampling (baseline levels, stress-induced levels at 15 min, stress-induced levels at 30 min), body condition, hatching date, nest score, and all interactions on corticosterone levels.

Dependent variables	Independent variables	df	*F*	*p*
A				
CORT levels	Age	**2,126**	**57.79**	**<0.01**
	Time	**2,126**	**553.23**	**<0.01**
	Hatching date	1,126	<0.01	0.99
	Nest score	1,124	0.51	0.48
	Body condition	**1,126**	**12.72**	**<0.01**
	Age × Time	**4,126**	**11.24**	**<0.01**
	Age × Hatching date	**2,126**	**4.10**	**0.02**
	Time × Hatching date	2,126	0.62	0.54
	Age × Nest score	2,120	0.61	0.54
	Time × Nest score	2,122	1.06	0.35
	Age × Body condition	2,124	0.15	0.86
	Time × Body condition	**2,126**	**6.37**	**<0.01**
	Age × Time × Hatching date	**4,126**	**2.78**	**0.03**
	Age × Time × Nest score	4,112	1.20	0.32
	Age × Time × Body condition	4,116	1.45	0.22
B				
CORT levels	Age	**2,133**	**60.83**	**<0.01**
	Time	**2,133**	**687.57**	**<0.01**
	Hatching date	1,133	1.24	0.27
	Nest score	1,131	0.37	0.55
	Body condition	1,133	13.08	**<0.01**
	Age × Time	**4,133**	**11.81**	**<0.01**
	Age × Hatching date	2,131	1.82	0.17
	Time × Hatching date	2,127	0.21	0.81
	Age × Nest score	2,125	0.22	0.80
	Time × Nest score	2,129	0.69	0.51
	Age × Body condition	2,123	0.16	0.85
	Time × Body condition	**2,133**	**6.93**	**<0.01**
	Age × Time × Hatching date	4,119	1.31	0.27
	Age × Time × Nest score	4,111	0.84	0.50
	Age × Time × Body condition	4,115	1.18	0.32

In this Table, (A) includes all data, while (B) includes all data but a statistical outlier with a very high corticosterone value. Bold fonts represent significant ($p < 0.05$) variables.

In addition, a second set of models were used to see if this repeatability could be related to the repeatability of body condition within individuals. In these additional models, the body condition index was added as an explanatory variable. In our data set, there was one data point with a very high corticosterone value (stress-induced corticosterone levels at 15 min: 60.15 ng/mL), which was potentially a statistical outlier. All statistics were run with and without this data point to make sure it did not affect our results and our interpretations.

Results

Age and the corticosterone stress response

Corticosterone levels were significantly linked to the "time factor" and corticosterone levels increased during the restraint stress protocol [$F_{(2,137)}$ = 483.67, $p < 0.01$; **Figures 1A–C**]. Importantly, corticosterone levels were also linked to the age of the chick [$F_{(2,137)}$ = 55.62, $p < 0.01$] and to the "age × time" interaction [$F_{(4,137)}$ = 9.52, $p < 0.01$], demonstrating that the corticosterone stress response was significantly correlated with the age of the chick (**Figure 1**). Specifically, baseline corticosterone levels did not significantly differ between 11-days old, 20-days old, and 37-days old chicks (*post-hoc* tests: all $p > 0.30$; **Figures 1D–F**). In contrast, stress-induced corticosterone levels at 15 min were higher in 11-days old chicks relative to 20-days old and 37-days old chicks (*post hoc* tests: all $p < 0.01$; **Figures 1D–F**) although stress-induced corticosterone levels at 15 min did not statistically differ between 20-days old and 37-days old chicks (*post-hoc* test: t = 1.01, p = 0.31; **Figures 1E,F**). Similarly, stress-induced corticosterone levels at 30 min were higher in 11 days old chicks relative to 20-days old and 37-days old chicks (*post-hoc* tests: all $p < 0.01$; **Figures 1D–F**) although stress-induced corticosterone levels at 30 min did not statistically differ between 20-days old and 37-days old chicks (*post-hoc* test: t = 0.97, p = 0.33; **Figures 1E,F**). The results remained similar if the potential statistical outlier was removed.

When comparing the corticosterone stress response between chicks and adults, corticosterone levels were significantly linked to the "time factor" [$F_{(1,114)}$ = 1434.22, $p < 0.01$], the "status" factor [$F_{(4,114)}$ = 10.12, $p < 0.01$] and their interaction [$F_{(4,114)} = 6.15, p < 0.01$], demonstrating that the corticosterone stress response significantly differed between individual statuses (11-, 20-, and 37-days old chick, and incubating and chick-rearing adults). For all statuses, corticosterone levels increased in response to the stress protocol. Baseline corticosterone levels did not differ between statuses (*post-hoc* tests: all $p > 0.200$). Stress-induced corticosterone levels of incubating and chick-rearing adults at 30 min were lower than those of the 11-days old chicks (*post-hoc* tests: all $p < 0.05$; **Figure 2**) but higher than those of 20- and 37-days old chicks (*post-hoc* tests: all $p < 0.05$; **Figure 2**). Stress-induced corticosterone levels of incubating and chick-rearing adults were similar (*post-hoc* test: p = 0.90; **Figure 2**). The results remained unchanged if the potential statistical outlier was removed.

Body condition and corticosterone levels

Corticosterone levels were significantly linked to the "time factor" [$F_{(2,134)}$ = 525,88 $p < 0.01$], to the age of the chick [$F_{(2,134)}$ = 56.17, $p < 0.01$] and to the "age × time" interaction [$F_{(4,134)}$ = 10.70, $p < 0.01$]. In addition, corticosterone levels were linked to the body condition index [$F_{(1,134)}$ = 9.79, $p < 0.01$] and the "body condition index × time" interaction [$F_{(2,134)}$ = 5.32, $p < 0.01$], demonstrating that the correlation between body condition on corticosterone levels varied between baseline and stress-induced levels. Corticosterone levels were not significantly linked to any other interaction (all $p > 0.60$). Specifically, baseline corticosterone levels were not significantly correlated with the body condition index (t = 0.13, p = 0.90; **Figure 3**) while stress -induced corticosterone levels were negatively correlated with the body condition index (stress-induced corticosterone levels at 15 min: $t = -3.06$, $p < 0.01$; stress-induced corticosterone levels at 30 min: $t = -3.63$, $p < 0.01$; **Figure 3**). Corticosterone levels were not significantly linked to the "body condition × age" and the "body condition index × age × time" interactions (all $p > 0.40$), demonstrating that the correlation between body condition on corticosterone levels did not differ between 11-, 20-, and 37-days old chicks. The results remained similar if the potential statistical outlier was removed.

Impact of multiple captures on the corticosterone stress response

In 20- and 37-days old chicks, corticosterone levels were linked to the "time factor" [$F_{(2,97)}$ = 527.81, $p < 0.01$] and to the age of the chick [$F_{(1,97)}$ = 4.23, p = 0.04] but not to the "age × time" interaction [$F_{(2,92)}$ = 0.81, p = 0.45]. Corticosterone levels were also linked to the body condition index [$F_{(1,97)}$ = 16.61, $p < 0.01$], and to the "body condition index × time" interaction [$F_{(2,97)}$ = 7.16, $p < 0.01$]. However, corticosterone levels were not significantly linked to the "previous capture" factor [$F_{(1,96)}$ = 0.01, p = 0.93, **Figure 4**], and any other interactions (all $p > 0.10$). The results remained similar if the potential statistical outlier was removed.

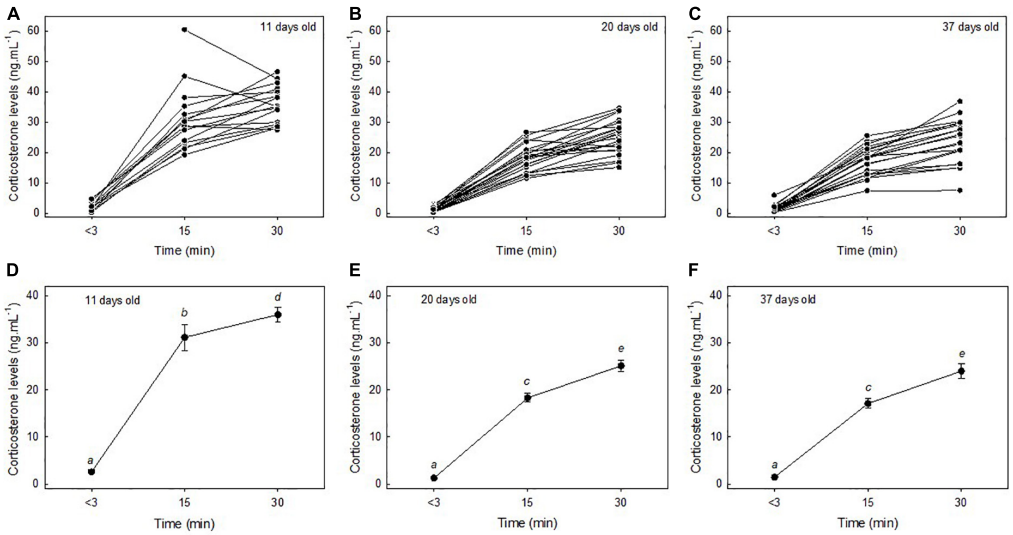

FIGURE 1
Inter-individual variability of the corticosterone stress response in snow petrel chicks at 11-days old **(A)**, 20-days old **(B)**, 37-days old **(C)**, and influence of the age of the chick on the corticosterone stress response **(D–F)**. Mean ± SE are represented and different letters indicate significant differences. Note that one data point with very high corticosterone levels was potentially a statistical outlier (stress-induced corticosterone levels at 15 min in 11-days old chicks: >60 ng/mL). All statistical tests remained similar if this data point was omitted from the data set.

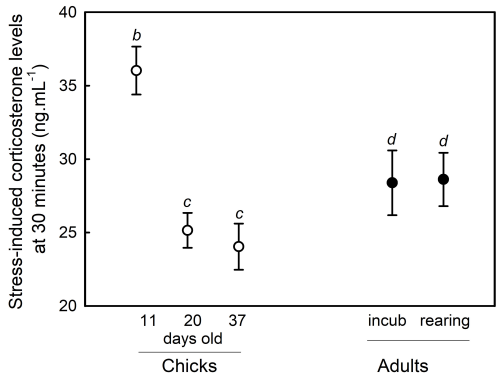

FIGURE 2
Difference in stress-induced corticosterone levels (after 30 min of restraint) between 11-days old chicks, 20-days old chicks, 37-days old chicks, incubating adults, and chick-rearing adults. Mean ± SE are represented and different letters indicate significant differences. White and black symbols represent chicks and adults, respectively.

Nest score, hatching date, and corticosterone levels

Although corticosterone levels were linked to the "time factor," the age of the chick, body condition, and the "age × time" and "time × body condition" interactions (Table 1), they were not linked to the nest score or any other interaction including the nest score (Table 1). Similarly, corticosterone levels were not significantly linked to hatching date or the "hatching date × time" interaction (Table 1A). There was a significant trend for an effect of the "hatching date × time" and the "hatching date × age × time" interactions (Table 1A), but this was the result of the potential statistical outlier and these interactions were far from being significant when this data point was removed (Table 1B).

Parental traits and corticosterone levels

Although corticosterone levels were linked to the "time factor," the age of the chick, body condition, and to the "age × time" and "time × body condition" interactions (Table 2), they were not linked to parental body condition, parental baseline corticosterone levels, parental stress-induced corticosterone levels or any interaction (Table 2). The results remained unchanged if the potential statistical outlier was removed (no parental data was available for this specific data point).

Repeatability of corticosterone levels and body condition

Baseline corticosterone levels were not repeatable (repeatability: $r \pm se = 0 \pm 0.12$, Figure 5). However, stress-induced corticosterone levels at 15 min were repeatable

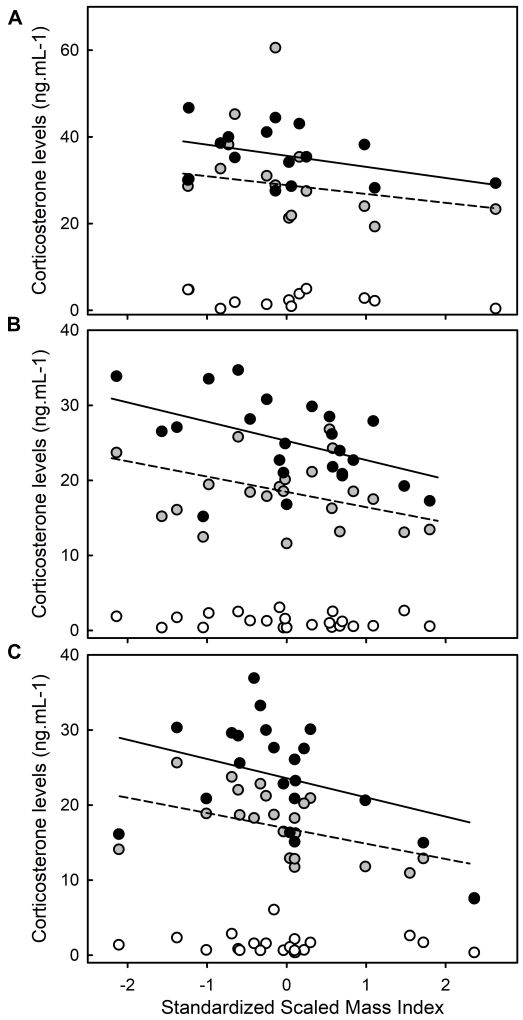

FIGURE 3
Influence of body condition (Scaled Mass Index) on corticosterone levels in 11-days old **(A)**, 20-days old **(B)**, and 37-days old **(C)** chicks. White, gray and black dots represent baseline corticosterone levels, stress-induced corticosterone levels after a 15 min restraint, and stress-induced corticosterone levels after a 30 min restraint, respectively. Solid and dashed lines represent significant relationships between body condition and corticosterone levels (dashed: stress-induced corticosterone levels at 15 min; solid: stress-induced corticosterone levels at 30 min).

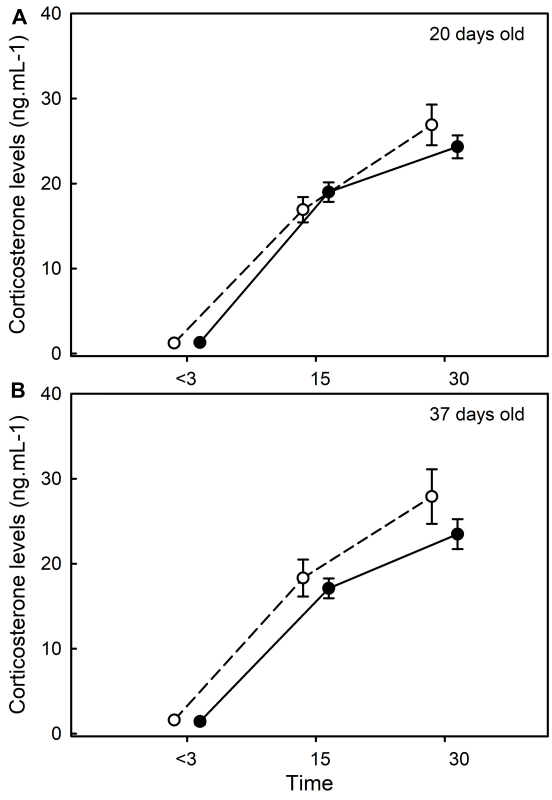

FIGURE 4
Influence of previous exposure to stress (i.e., capture/restraint protocol) on the corticosterone stress response of 20-days old **(A)** and 37-days old **(B)** chicks. Mean ± SE are represented and no significant differences were found. Black and white dots represent the petrels that have been previously captured or not, respectively.

(repeatability: $r = 0.23 \pm 0.16$, $r = 0.38 \pm 0.16$ if the potential statistical outlier was removed, Figure 5). Stress-induced corticosterone levels at 30 min were also repeatable (repeatability: 30 min, $r = 0.57 \pm 0.13$, Figure 5). Body condition was also repeatable ($r = 0.51 \pm 0.14$). The repeatability of corticosterone levels was reduced when the body condition index was included in the models testing the repeatability of corticosterone levels. Specifically, baseline corticosterone levels were still not repeatable (repeatability: $r \pm se = 0 \pm 0.12$). Stress-induced corticosterone levels at 15 min were not repeatable (repeatability: $r = 0 \pm 0.10$, $r = 0.01 \pm 0.12$ if the potential statistical outlier was removed), while stress-induced corticosterone levels at 30 min remain repeatable (repeatability: 30 min, $r = 0.43 \pm 0.16$).

Discussion

In this study, we reported not only a strong heterogeneity of the corticosterone stress response at all ages in snow petrel chicks, but also a high repeatability of this corticosterone stress response throughout the post-hatching developmental period (e.g., repeatability: $r = 0.57$ for stress-induced corticosterone levels after a 30 min restraint). This suggests that this stress response is a consistent individual trait that shows a high inter-individual variability. Although this corticosterone stress response is linked to the internal state of the chick (age, body condition), we however did not find any strong evidence linking parental traits

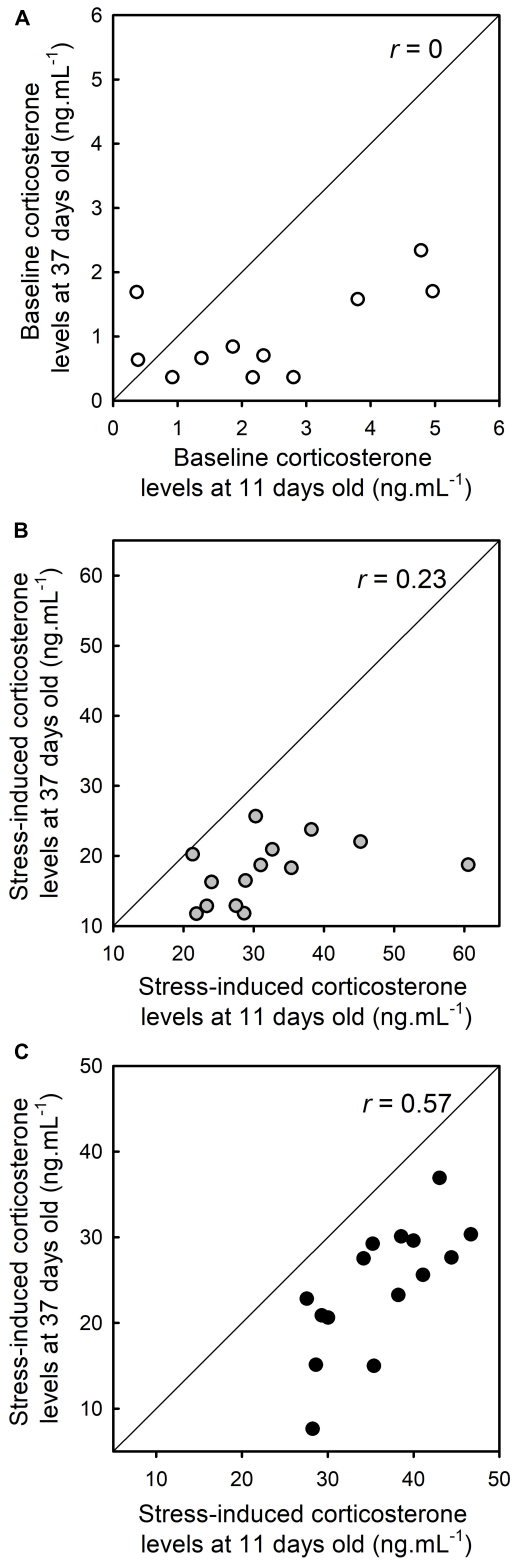

FIGURE 5
Repeatability (r) of baseline corticosterone levels **(A)**, stress-induced corticosterone levels after a 15 min restraint **(B)**, and stress-induced corticosterone levels after a 30 min restraint **(C)**.

(parental condition or parental corticosterone stress response) and other ecological factors (nest quality, hatching date, previous exposure to sampling) with the chick corticosterone stress response. In addition, we found that the magnitude of the corticosterone stress response of snow petrel chicks is similar if not higher than that of breeding adults, suggesting that the chicks from this species do not show a stress hyporesponsive period at young ages [*sensu* Wada (2008)], as commonly found in altricial species. Our results emphasize that the corticosterone stress response shows a high-interindividual variability and future long-term studies are now required to understand whether this heterogeneity may play a role in the ability of developing individuals to cope with the perturbations that occur in Antarctica.

Baseline corticosterone levels

We found that baseline corticosterone levels were not affected by the age of the chick and they did not significantly differ between chicks and adults. In addition, baseline corticosterone levels of chicks were not repeatable throughout the post-hatching phase. These results are consistent with previous studies, which have shown that baseline corticosterone levels are labile and poorly repeatable [Romero and Reed, 2008; Rensel and Schoech, 2011; but see Ouyang et al. (2011)]. In their meta-analysis, Taff et al. (2018) reported that the repeatability of baseline corticosterone is low (r = 0.18, CI = 0.11–0.24) and we may have lacked statistical power to detect such a low repeatability in our study (sample size of 15 snow petrel chicks). In addition, baseline corticosterone levels were not related to body condition at any age (11-, 20-, and 37-days old). However, baseline corticosterone levels are classically elevated when individuals are in poor condition, as demonstrated in many bird species [e.g., adults: Lynn et al. (2003, 2010)], including seabirds [adults and chicks; e.g., Kitaysky et al. (2001) and Quilfeldt et al. (2006)], and even adult and chick snow petrels (Angelier et al., 2009, 2015; Dupont et al., 2021). Therefore, our results suggest that snow petrel chicks were not energetically constrained during the 2012 breeding season and this interpretation is indeed supported by the high fledging success of the 2012 season [see Sauser et al. (2021)]. Supporting further this interpretation, baseline corticosterone levels were not correlated with hatching date, and we did not find any evidence that baseline corticosterone levels were higher when the chicks were growing in a less protected nest. Inclement weather is known to increase baseline corticosterone levels in birds (Bize et al., 2010; de Bruijn and Romero, 2018; Crino et al., 2020) and hatching date and nest characteristics are known to be linked with local weather and fledging success. Specifically, the weather usually deteriorates as the breeding

season progresses (snow fall and strong wind) and chicks from more sheltered nest are less affected by inclement weather (Dupont et al., 2020; Sauser et al., 2021). However, this was not the case in 2012, which was characterized by very little snow fall and no strong wind during the chick-rearing period (Sauser et al., 2021), and as a result, all chicks survived in our study. This specific mild weather may explain why baseline corticosterone levels were unrelated to nest score and hatching date.

Ontogeny of the corticosterone stress response during the post-hatching phase

We found that the chicks elicit a strong corticosterone stress response when they are as young as 11-days old. Indeed, the corticosterone stress response of 11-days old chicks is even greater than the corticosterone stress response of older chicks or even adults. This demonstrates that the HPA axis is fully operational at a very young age in this altricial seabird species. Although this result is not supported by several studies (Sims and Holberton, 2000; Love et al., 2003; Wada et al., 2007, 2009; Rensel et al., 2010; Bebus et al., 2020; Jones et al., 2021), two studies found similar results: Adams et al. (2008) found in another petrel species that chicks elicit a significant corticosterone stress response a few days after hatching. Similarly, Berg et al. (2019) recently found the same pattern in another altricial species, the green-rumped parrotlet. In our study, we sampled the chicks 11 days after hatching and it is possible that the HPA axis was not active before that age. However, 11 days represent only a fourth of the post-hatching developmental phase in the snow petrel and our results therefore demonstrate that snow petrel chicks are able to elicit a fully operational corticosterone stress response at a young age. It remains unclear why the ontogeny of the corticosterone stress response is rapid in a few altricial species (Adams et al., 2008; Berg et al., 2019; this study) but not others (Sims and Holberton, 2000; Love et al., 2003; Wada et al., 2007, 2009; Rensel et al., 2010; Bebus et al., 2020; Jones et al., 2021). A dampened stress response in young altricial chicks is thought to minimize the costs of bearing elevated corticosterone levels [slower growth, altered immunity, etc.; Kitaysky et al. (2003) and Martin et al. (2005)], but a functional HPA axis may be necessary to activate metabolic functions (Jimeno et al., 2018, 2020), which is crucial in precocial species and in a few altricial species living under extreme weather conditions such as the snow petrel. This could explain our results because snow petrel chicks can be left unattended by their parents at a very young age (Barbraud et al., 1999). Indeed, snow petrels, as many other seabird species, are often considered as semi-altricial because their chicks are covered with down at hatching and are able to maintain their body temperature at high levels soon after hatching. In our study, we measured the corticosterone stress response through plasma levels of corticosterone in response to a standardized stress protocol. Because the action of corticosterone is mediated through the binding to multiple types of receptors (Landys et al., 2006), it would be necessary to measure the location and the density of these glucocorticoid receptors to fully understand how the corticosterone stress response is influenced by age and post-hatching conditions. However, for ethical reasons (i.e., the study of receptors requires to euthanize the birds to collect organs), it was obviously not possible to study this question in long-lived Antarctic snow petrels.

We found in our study that snow petrel chicks show a high inter-individual variability in the magnitude of the corticosterone stress response (i.e., stress-induced corticosterone levels at 15 and 30 min). Importantly, we also reported for the first time in a wild bird species that this corticosterone stress response is repeatable throughout the post-hatching phase. Previous studies have reported that the corticosterone stress response is repeatable within individuals but most of these studies have focused on the adult life [reviewed in Taff et al. (2018)]. Interestingly, our repeatability coefficient is within the upper range that was reported by Taff et al. (2018) in their meta-analysis of dozens of species [i.e., Taff et al. (2018): $r = 0.38$, CI = 0.29–0.45], even after considering the potential effect of body condition on this repeatability measurement. This suggests that the corticosterone stress response could be a consistent physiological trait in snow petrels, as in many other bird species. In addition, this repeatability of the corticosterone stress response could also result from the circumstances in which the chicks are found rather than from a consistency of this physiological trait. Supporting this possibility, we found that the body condition of chicks is also repeatable through development (i.e., from 11-days old to 37-days old) and is correlated to the corticosterone stress response. Indeed, the corticosterone stress response of chicks is certainly determined simultaneously by the circumstances (i.e., rearing environment) and a genetic component (Jenkins et al., 2014) that both translate into a high repeatability of stress-induced corticosterone levels in our study. Supporting this idea, we found that body condition accounts for a part, but not all, of the repeatability of stress-induced corticosterone levels. This is consistent with the idea that the corticosterone stress response and the functioning of the HPA axis may be subject to selection (Angelier and Wingfield, 2013; Jenkins et al., 2014; Vitousek et al., 2014) and at least partly heritable (Satterlee and Johnson, 1988; Evans et al., 2006; Almasi et al., 2010; Angelier et al., 2011; Baugh et al., 2012; Jenkins et al., 2014; Béziers et al., 2019). Surprisingly, we did not find any evidence of such heritability in our study because the corticosterone stress response of the chick was not related to the corticosterone stress response of the parent.

TABLE 2 Model selections to test the influence of the age of the chick (11-, 20-, and 37-days old), body condition of the chick, baseline and stress-induced corticosterone levels of the parent ("baseline CORT" and "stress-induced CORT"), body condition of the parent ("parental body condition"), and all interactions on corticosterone levels.

Dependent variables	Independent variables	df	*F*	*p*
CORT levels	Age	**2.93**	**51.17**	**<0.01**
	Time	**2.93**	**522.53**	**<0.01**
	Body condition	**1.93**	**16.82**	**<0.01**
	Parental body condition	1.93	0.40	0.53
	Baseline CORT	1.93	0.55	0.46
	Stress-induced CORT	1.93	0.94	0.33
	Age × Time	**4.93**	**9.74**	**<0.01**
	Age × Body condition	2.85	1.73	0.18
	Time × Body condition	**2.93**	**9.67**	**<0.01**
	Age × Parental body condition	2.81	0.20	0.82
	Time × Parental body condition	2.89	1.37	0.26
	Age × Baseline CORT	2.83	1.84	0.17
	Time × Baseline CORT	2.91	2.10	0.13
	Age × Stress-induced CORT	2.79	0.04	0.96
	Time × Stress-induced CORT	2.87	0.47	0.63
	Age × Time × Body condition	4.75	2.37	0.06
	Age × Time × Parental body condition	4.63	0.59	0.67
	Age × Time × Baseline CORT	4.67	1.58	0.19
	Age × Time × Stress-induced CORT	4.71	0.43	0.79

Bold font represents significant ($p < 0.05$) variables.

However, we only sampled one of the two parents to limit nest disturbance, and the parents were sampled during the chick-rearing phase when they are known to actively modulate their corticosterone stress response (Angelier et al., 2015, 2020), limiting therefore our ability to test the heritability of this stress response. To fully test the heritability of the corticosterone stress response, it would have been necessary to sample the parents and their chicks at the same life-history stage, but it is unpractical because the chicks return to the colony to breed only when they are 10 years old by average (Chastel et al., 1993).

Ecological determinants of the corticosterone stress response

As expected, we found that stress-induced corticosterone levels were affected by the internal state of the chicks. Specifically, the age of the chick had a strong influence on stress-induced corticosterone levels and chicks had higher stress-induced corticosterone levels at 11-days old relative to 20- or 37-days old. This difference may be explained by a smaller amount of body reserves at younger age because the ability of smaller chicks to store body reserves is usually limited by their smaller structural size. Indeed, the corticosterone stress response has been convincingly linked to the nutritional status of chicks in multiple species (e.g., Sockman and Schwabl, 2001), including seabirds (e.g., Kitaysky et al., 2001; Quilfeldt et al., 2006) and snow petrels (Dupont et al., 2021). Supporting further this idea, we found that stress-induced corticosterone levels were negatively correlated with body condition in 11-, 20-, and 37-days old chicks although it remains to be determined if this correlation results from a satiety effect (low corticosterone levels in recently fed chicks) or an effect of nutritional reserves (low corticosterone levels in chick with large body reserves).

In contrast, and contrary to our prediction, we did not find any evidence supporting an effect of nest quality on the corticosterone stress response. However, nest quality influences the exposure of snow petrel chicks to inclement weather and predation risk, two factors that could affect the corticosterone stress response [Cockrem and Silverin, 2002; de Bruijn and Romero, 2018; Crino et al., 2020; but see Rivers et al. (2011)]. This suggests that most of the inter-individual variability in the corticosterone stress response may be relatively independent of these specific abiotic factors during the post-hatching phase (at least in the study species). Instead, the corticosterone stress response may rather be an individually repeatable trait [reviewed in Taff et al. (2018)], which is determined by a genetic component and/or the nutritional status of the chick [see also Jenkins et al. (2014)]. Supporting this idea, we found that the corticosterone stress response was also independent of two proxies of parental quality (parental body condition and hatching date). More successful parents usually breed earlier

and are in better condition in snow petrels (Barbraud and Chastel, 1999; Sauser et al., 2021), but hatching date and parental body condition were not related to the chick's corticosterone stress response at any age in our study. Finally, we did not find any evidence for an effect of repeated exposure to capture and handling on the chick's corticosterone stress response (Cyr and Romero, 2009), or on baseline corticosterone levels. This suggests that habituation of the corticosterone stress response is unlikely to occur in snow petrel chicks and that repeated capture and sampling may not detrimentally affect the development of the chicks in this petrel species [see also Dupont et al. (2020)]. Supporting this idea, all chicks successfully fledged in our study, suggesting that the fitness impact of repeated capture and sampling is probably limited, at least when environmental conditions are favorable [as it was the case during that breeding season, Sauser et al. (2021)]. However, we must remain cautious because our sample size of unmanipulated petrels was relatively limited (n = 5 for 20-days old petrels, n = 7 for 37 days old petrels). From a mechanistic point of view, repeated exposure to acute stressors may affect the functioning of the HPA axis in vertebrates (Cyr and Romero, 2009) but our results suggest that habituation and sensitization of the corticosterone stress response do not occur at that age in snow petrel chicks, except possibly in case of frequent exposure to stress [see Sagar et al. (2019)] but we could not test this because of limited sample size. Again, this argues for the idea that the corticosterone stress response is probably an individually consistent trait that shows a strong inter-individual variability during the post-hatching developmental phase.

Data availability statement

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

Ethics statement

Licences and permissions were granted by the Ethic Committee of the Institut Polaire Francais (IPEV).

Author contributions

FA: conceived the research, conducted field work, analyzed the data, and wrote the manuscript. JW and OC: conceived the research and provided comments on the manuscript. CB: provided comments on the manuscript. CP: conducted laboratory work and field work. CT: conducted laboratory work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.902202/full#supplementary-material

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Individual variation, personality, and the ability of animals to cope with climate change

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The Sixth Assessment of the Intergovernmental Panel on Climate Change describes negative effects of climate change on animals occurring on a larger scale than previously appreciated. Animal species are increasingly experiencing more frequent and extreme weather in comparison with conditions in which the species evolved. Individual variation in behavioural and physiological responses of animals to stimuli from the environment is ubiquitous across all species. Populations with relatively high levels of individual variation are more likely to be able to survive in a range of environmental conditions and cope with climate change than populations with low levels of variation. Behavioural and physiological responses are linked in animals, and personality can be defined as consistent individual behavioural and physiological responses of animals to changes in their immediate environment. Glucocorticoids (cortisol and corticosterone) are hormones that, in addition to metabolic roles, are released when the neuroendocrine stress system is activated in response to stimuli from the environment perceived to be threatening. The size of a glucocorticoid response of an animal is an indication of the animal's personality. Animals with reactive personalities have relatively high glucocorticoid responses, are relatively slow and thorough to explore new situations, and are more flexible and able to cope with changing or unpredictable conditions than animals with proactive personalities. Animals with reactive personalities are likely to be better able to cope with environmental changes due to climate change than animals with proactive personalities. A reaction norm shows the relationship between phenotype and environmental conditions, with the slope of a reaction norm for an individual animal a measure of phenotypic plasticity. If reaction norm slopes are not parallel, there is individual variation in plasticity. Populations with relatively high individual variation in plasticity of reaction norms will have more animals that can adjust to a new situation than populations with little variation in plasticity, so are more likely to persist as environments change due to climate change. Future studies of individual variation in plasticity of responses to changing environments will help understanding of how populations of animals may be able to cope with climate change.

KEYWORDS

individual variation, personality, climate change, reaction norms, glucocorticoids, HIREC, phenotypic plasticity

Introduction

Environmental conditions are changing throughout the world due to climate change and other anthropogenic causes such as destruction of animal habitats. Changes in weather patterns associated with climate change include increased temperatures, both increases and decreases in rainfall, and increased frequency of storms. Individual animals may be able to cope with climate change by making behavioural changes and physiological adjustments that enable them to stay in their current range, or they may be able to move and survive in new areas. Animals with these characteristics may be favoured by natural selection. The ability of populations of animals to cope with climate change thus depends on individual variation in responses of animals to environmental stimuli, and on the extent to which individual animals are plastic and able to change their responses.

The central role of individual variation in the success of populations of animals in coping with climate change is explored in this review. An overview of the effects of climate change on animal populations is followed by a consideration of individual variation in responses of animals to changes in their environment, and of individual variation in plasticity in responses. The importance of individual characteristics known as personality in determining behavioural and physiological responses of animals and fitness is considered, including relationships between glucocorticoid responses, personality, and fitness. Animals with reactive personality styles are likely to be better at coping with climate change than proactive animals. A reaction norm approach enables identification of individual variation in plasticity of responses of animals to changing environments, and the last section of the review considers the use of reaction norms to help understand how populations of animals may cope with climate change.

Climate change

Climate change

It is important to define climate and to distinguish between climate and weather when considering climate change. Weather refers to atmospheric conditions such as wind, temperature, cloudiness, rainfall, humidity, and air pressure at a given time, while climate refers to average weather conditions over long periods of time (National Oceanic and Atmospheric Administration [NOAA], 2021). A standard period for averaging these variables is 30 years (World Meteorological Organization[1]).

Carbon dioxide, methane and nitrous oxide are together known as greenhouse gases. Greenhouse gas emissions due to human activities have increased since the pre-industrial period in the 1700s, leading to atmospheric concentrations of greenhouse gases that are unprecedented in at least the last 800 000 years. The increased concentrations of greenhouse gases, especially carbon dioxide, are causing changes in the Earth's energy budget that in turn are leading to warming of the global climate (Intergovernmental Panel on Climate Change [IPCC], 2014). Changes in the global climate system include warming of the atmosphere with increased land and ocean temperatures, reductions in the Greenland and Arctic ice sheets, shrinkage of glaciers in the Northern and Southern Hemispheres, and sea level rise (Intergovernmental Panel on Climate Change [IPCC], 2014). These changes are commonly called climate change. Climate change, caused by anthropogenic increases in atmospheric greenhouse gases, is a combination of long-term changes in climate and short-term changes in the frequency and intensity of weather events that can have high impacts on local areas and on animals (see **Figure 1**). The Framework Convention on Climate Change (UNFCCC) defines climate change as: "a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is in addition to natural climate variability observed over comparable time periods" (United Nations, 1992). Another term for climate change, human-induced rapid environmental change (HIREC), refers to environmental changes caused by human activities that are occurring more rapidly and at larger scales than environmental changes that organisms have likely experienced in their evolutionary past (Sih et al., 2010).

The Intergovernmental Panel on Climate Change (IPCC) was established in 1988 and is the international body for the assessment of science related to climate change. While the IPCC includes changes due to natural processes in its definition of climate change, in practice IPCC considerations are focused on changes due to anthropogenic greenhouse gas emissions. The IPCC is now (2022) in its Sixth Assessment cycle (Intergovernmental Panel on Climate Change [IPCC], 2021b). The report from Working Group I (the Physical Science Basis) was published in August 2021 (Intergovernmental Panel on Climate Change [IPCC], 2021a) and the report from Working Group II (Impacts, Adaptation and Vulnerability) was published in February 2022 (Intergovernmental Panel on Climate Change [IPCC], 2021b). A third working group report (Mitigation of Climate Change), and the AR6 Synthesis Report, will be published later in 2022.

The Sixth Assessment explores the likely consequences for the world climate of five scenarios for emissions of greenhouse gases. Global surface temperature will continue to increase until at least mid-century under all scenarios. Global warming will reach 1.5°C by the early 2030s and will reach 2°C by mid-century unless there are deep reductions in carbon dioxide and other greenhouse gas emissions (Intergovernmental Panel on Climate Change [IPCC], 2021a). While it remains possible for emissions to be reduced to keep warming below 1.5°C, measures

1 https://public.wmo.int/en/our-mandate/climate

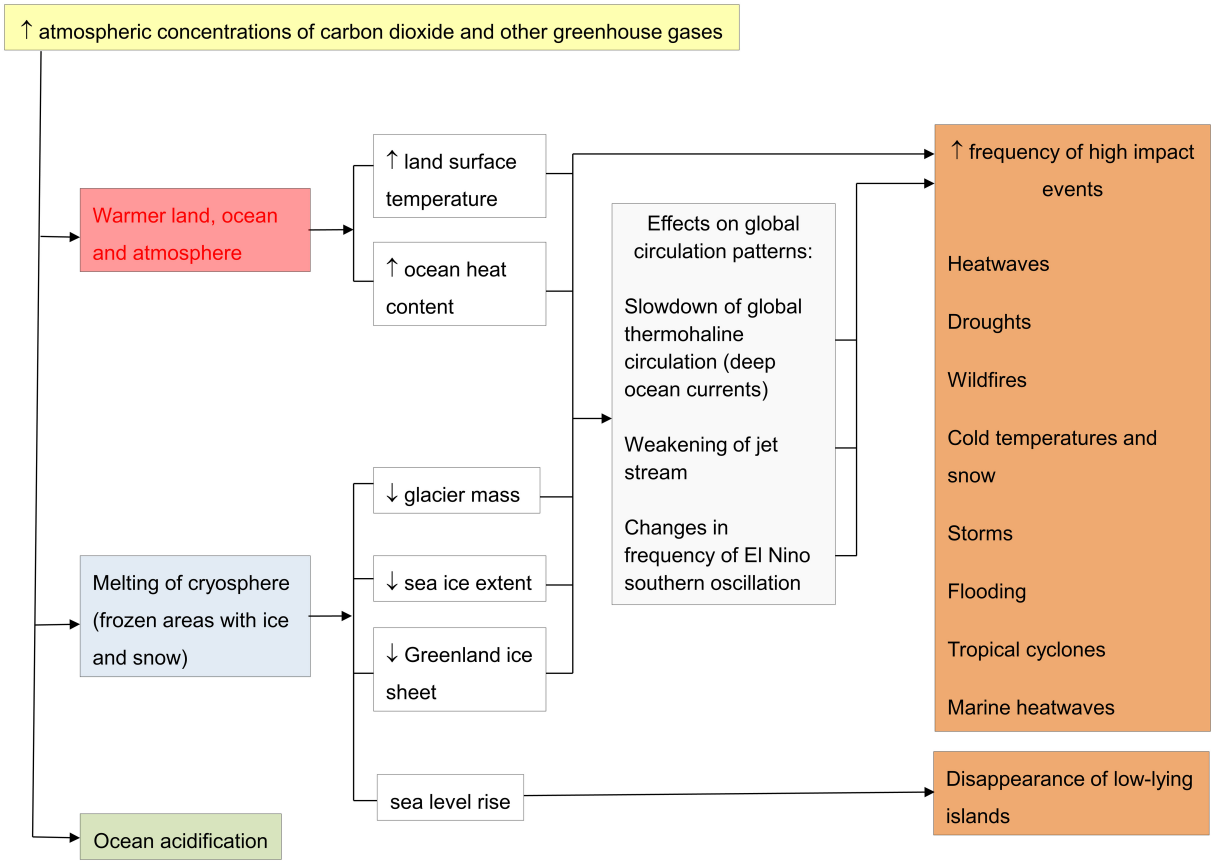

FIGURE 1
Effects of increasing atmospheric concentrations of carbon dioxide and other greenhouse gases on climate and weather [based on (World Meteorological Organization, 2021)].

taken by governments to reduce national emissions have thus far not been sufficient to achieve this goal.

The Sixth Assessment states that climate change has altered marine, terrestrial and freshwater ecosystems all around the world, and that the effects of climate change have been experienced earlier, are more widespread, have been of greater magnitude, and are having more far-reaching consequences than previously anticipated (Intergovernmental Panel on Climate Change [IPCC], 2022b). It is predicted that magnitude of extreme conditions will become greater and previously rare weather conditions will become more common in coming decades (Intergovernmental Panel on Climate Change [IPCC], 2021a). Hot conditions on land have become more frequent and intense since the 1950s, while cold extremes have become less frequent. Terrestrial heatwaves that exceed the physiological thresholds of some species are regularly occurring, and marine heatwaves with generally negative consequences for animals (Cheung and Frölicher, 2020) are becoming more frequent (Intergovernmental Panel on Climate Change [IPCC], 2022a). Heavy precipitation (rainfall and snowfall) and storm events are more common and intense, and sea levels are rising (Intergovernmental Panel on Climate Change [IPCC], 2021a).

Effects of climate change on animals

Long term studies of responses of free-living animals to changes in climate variables generally identify the responses as being due to climate change. For example, Charmantier et al. (2008) reported an advance of 14 days over 47 years in the timing of egg laying in a great tit (*Parus major*) population in the United Kingdom. The advance in laying date was attributed to increased spring temperatures due to climate change leading to earlier emergence of caterpillars which are the primary food for nestlings. Similarly, advances of more than three days per decade in the timing of egg laying in two blue tit (*Cyanistes caeruleus*) populations in evergreen forest on Corsica in the Mediterranean corresponded with changes in temperature attributed to climate change (Bonamour et al., 2019).

The IPCC Sixth Assessment reported that, for terrestrial, freshwater, and marine animals and plants reported to be

affected by climate change, half to two-thirds of the species have shifted their ranges to higher latitudes, and approximately two-thirds of species had advanced their timing of spring activities (Intergovernmental Panel on Climate Change [IPCC], 2022b). Responses of animals, including changes in physiology, geographic range and shifting seasonal timing are often not sufficient to cope with recent climate change. Foden et al. (2013) considered that 11 to 15% of amphibian species and 6 to 9% of bird species are both highly climate change vulnerable and already threatened with extinction (listed on the IUCN Red List). The Sixth Assessment considered that a median of 9% and a maximum of 14% of assessed terrestrial species were likely to face a very high risk of extinction (equivalent to the IUCN critically endangered status) at global warming of 1.5°C (Intergovernmental Panel on Climate Change [IPCC], 2022b). This level of warming will be reached in the 2030s if emissions of greenhouse gases continue at their current rate. Even if emissions are markedly reduced in coming years, there will be ongoing local population extinctions and extinctions of species due to climate change.

Local extinctions due to climate change were detected in 47% of plant and animal species examined, with local extinctions more prevalent in tropical than temperate habitats (Wiens, 2016; Intergovernmental Panel on Climate Change [IPCC], 2022a). A survey of plant distribution in Arizona mountains found local extinctions of 15 species of plants, including *Quercus gambelii* (Gambel oak), *Muhlenbergia porteri* (bush muhly) and *Urochloa arizonica* (AZ panic grass), in comparison with 50 years earlier (Brusca et al., 2013). In the alpine Himalayas of Sikkim 75 species of plants, including *Rhododendron nivale* (dwarf snow rhododendron), *Potentilla fruticosa* (shrubby cinquefoil) and *Lepidium capitatum* (Himalayan peppergrass), were locally extinct in comparison with 1850 (Telwala et al., 2013). Species extinctions have been attributed to climate change for the golden toad (*Incilius periglenes*) that lived in cloud forest in Costa Rica and for the Bramble Cays Melomys (*Melomys rubicola*), an Australian rodent. It seems likely that other species living in remote areas will also have become extinct due to climate change.

It is also valuable to consider responses of animals to variables associated with climate change, with studies of these responses providing information that will help with prediction of effects of climate change on animals. **Table 1** gives examples of responses of animals to changes in climate and weather variables. A review of studies of behavioural responses of invertebrates and vertebrates to climate variables found that changes in phenology were the most commonly reported responses (Beever et al., 2017). Changes in the timing of reproductive behaviour were the most common timing changes, followed by changes in timing of movements (dispersal or migration). Changes in the timing of feeding, foraging, and habitat use were also reported, along with changes in thermoregulation behaviour activities. For the studies that were considered, temperature was identified in 67% of the studies as the climate variable associated with changes in behaviour. Some changes in animal behaviour were attributed to indirect

TABLE 1 Examples of responses of animals to changes in the environment associated with climate and weather variables.

Change in environment	Response	References
Warmer spring temperatures	Advance in timing of egg laying in blue tit (*Cyanistes caeruleus*) population in evergreen forest on Corsica in the Mediterranean.	Bonamour et al., 2019
	Advance of 14 days over 47 years in egg laying in great tit (*Parus major*) population in the United Kingdom. The advance in egg-laying was associated with earlier emergence of caterpillars which are the primary food for nestlings.	Charmantier et al., 2008
	Advance of 7.8 days over 42 years in timing of egg laying in Mandt's black guillemot (*Cepphus grylle mandtii*) in Alaska	Sauve et al., 2019
Marine heatwaves and changes in ocean currents	Change in foraging area of little penguins (*Eudyptula minor*) in year when ocean currents changed due to marine heatwave	Evans et al., 2020
High temperatures	Scaly feathered finch (*Sporopipes squamifrons*) and Kalahari scrub-robin (*Erythropygia paeana*) changed habitat use on days with higher than usual temperatures	Martin et al., 2015
Droughts	Increased drought intensity correlated with decreased occupancy of drought areas by North American breeding birds that lived in these areas	Cady et al., 2019
Increasing sea surface temperatures	45 species of fish had major changes in range in south-eastern Australia coastal waters since the 1980s	Last et al., 2011
Increasing air temperatures	Four of seven lizard species in Joshua Tree National Park in California showed range shifts in the last 50 years to occupy higher elevation habitats	Barrows et al., 2020
Rainfall becoming more sporadic in the Neotropics	Mortality of eggs laid in trees by treefrogs (*Dendropsophus ebraccatus*) in Panama has likely become greater over 40 years, with a change in rainfall patterns likely leading to changes in egg laying patterns with a greater proportion of eggs laid in water than before.	Touchon, 2012
Storms	Arctic storm led to belugas (*Delphinapterus leucas*) moving to another area	Scharffenberg et al., 2020

effects of climate variables such as changes in food resources or habitat structure.

Animal populations can persist in changing environments by individual animals changing their behaviour or their physiology, for example, by starting to feed at a different time of day or by increasing their evaporative heat loss in hot conditions, by moving to a different area, or by adapting through a change in the genetic composition of the population (Fuller et al., 2010). The ability of a population to cope with changing environments and persist in the face of climate change thus depends on the extent to which individual animals can adjust to the changes or move to a new area, and the extent to which the

TABLE 2 Examples of individual variation in behavioural and physiological variables.

Variable	Example of individual variation	References
Movement and locomotor activity	Locomotor activity and feeding activity in zebra finches (*Taeniopygia guttata*) introduced to a novel environment	Careau et al., 2020
Foraging	Foraging behaviour in brook charr (*Salvelinus fontinalis*) in Canada	Farwell et al., 2014
	Foraging strategies, foraging depth, foraging areas, and day or night foraging behaviour in Galapagos sea lions (*Zalophus wollebaeki*)	Schwarz et al., 2021
Home range	Home range and environmental niche of white storks (*Ciconia ciconia*) in Germany	Carlson et al., 2021
Migration	Review of individual variation in animal movements (foraging, regular daily movements, seasonal movements, annual migration, and erratic nomadic movements)	
	Timing and duration of migration of sea trout (*Salmo trutta*) between freshwater in Denmark and the sea in successive years	Birnie-Gauvin et al., 2021
	Migration date from New Zealand in bar-tailed godwits (*Limosa lapponica baueri*)	Conklin et al., 2013
	Timing, total duration and total distance of migration, as well as the location of individual wintering areas for common terns (*Sterna hirundo*) from a breeding colony in northwest Germany	Kurten et al., 2022
	Arrival time of northern wheatears (*Oenanthe oenanthe*) in spring in Sweden, and time between arrival and breeding	Low et al., 2019
Boldness in new situations	Vigilance, boldness, and investigating behaviours in arctic foxes (*Vulpes lagopus*) in Sweden	Choi et al., 2019
	Boldness in three-spined sticklebacks (*Gasterosteus aculeatus*)	Jolles et al., 2019
	Boldness in brown trout (*Salmo trutta*)	Kortet et al., 2014
	Boldness in brown anole lizards (*Anolis sagrei*) in Florida	Lapiedra et al., 2017
	Boldness in brushtail possums (*Trichosurus vulpecula*)	Mella et al., 2015
Exploration	Exploration tendency, behavioral flexibility, and aggressiveness in brown trout	Adriaenssens and Johnsson, 2011
	Exploration behaviour in European green lizards (*Lacerta viridis*)	Bajer et al., 2015
	Exploration, activity, and responses to restraint in Belding's ground squirrels (*Urocitellus beldingi*) in California	Dosmann et al., 2015
	Exploration behaviour in brown anole lizards (*Anolis sagrei*) in Florida	Lapiedra et al., 2017
	Exploration behaviour in juvenile European rabbits (*Oryctolagus cuniculus*)	Rodel et al., 2015
	Exploration behaviour and aggressiveness in wild boars (*Sus scrofa*) in Europe	Vetter et al., 2016
	Exploration behaviour in the western clawed frog [*Xenopus (Silurana) tropicalis*]	Videlier et al., 2014
Risk taking	Risk-taking in European green lizards (*Lacerta viridis*)	Bajer et al., 2015
	Risk taking behaviour in Namibian rock agamas (*Agama planiceps*)	Carter et al., 2012
Social behaviour	Behaviour of great tits in flocks	Aplin et al., 2014
Neophobia	Approach to novel food in Gouldian finch (*Erythrura gouldiae*)	Eccles et al., 2021
Glucocorticoid responses	Review that describes extensive individual variation in glucocorticoid responses to stimuli perceived as threatening for animals from all vertebrate groups	Cockrem, 2013b
	Corticosterone responses of little penguins (*Eudyptula minor*)	Cockrem et al., 2017
	Cortisol responses of goldfish (*Carassius auratus*)	Cockrem et al., 2019
	Corticosterone responses of brown tree snakes (*Boiga irregularis*)	Mathies et al., 2001
	Urinary corticosterone responses of cane toads (*Rhinella marina*)	Narayan et al., 2011
	Corticosterone responses of brown lemmings (*Lemmus trimucronatus*)	Romero et al., 2008
Reproductive hormones	Plasma testosterone response to GnRH in male dark-eyed juncos *(Junco hyemalis)*	Jawor et al., 2006
	Plasma testosterone in blue tits (*Cyanistes caeruleus*) during nest-building and egg-laying	Kempenaers et al., 2008
	Plasma estradiol-17b during egg formation in the European starling (*Sturnus vulgaris*)	Williams et al., 2004
Metabolic rate	Consistent individual differences in metabolic rate in various fish species	Metcalfe et al., 2016

population can adapt to the changes through natural selection of characteristics that enable individual animals to cope with a changed environment.

Individual variation

Individual variation in behavioural and physiological traits

Animals have morphological, physiological, and behavioural characteristics known as traits, for example, plumage colour in a bird, level of activation of the hypothalamo-pituitary-adrenal axis and glucocorticoid secretion in response to a threat, or timing of departure in a migratory species. For every trait, there are differences between individual animals in the level or nature of the trait. Individual differences arise from a combination of experiences of the animal and additive genetic variation (Dochtermann et al., 2015). Examples of genetic variation contributing to individual differences in glucocorticoid responses are the development of lines of rainbow trout (Overli et al., 2002) and Japanese quail (Odeh et al., 2003) selected for low or high glucocorticoid responses. Individual differences resulting from experiences of an animal include maternal and paternal effects, epigenetic effects, and environmental effects that can have long-term consequences (Dochtermann et al., 2015), such as prolonged periods of food shortage.

Some examples of individual variation in behavioural and physiological traits are given in **Table 2**. Recognition of individual variation, and of its significance, led Charles Darwin to formulate the concept of natural selection in which some animals have characteristics or traits that enable them to be more likely to survive, successfully reproduce and pass on heritable traits to their offspring, leading over time to these traits becoming more frequent in the population (Darwin, 1859). Natural selection, the process in which heritable differences between organisms in survival and reproduction lead to increases in the proportion of beneficial traits within a population from one generation to the next, is a fundamental concept for biology (Gregory, 2009). In other words, natural selection is a process in which some organisms have higher fitness than others (produce more offspring than others) and hence the genotypes of these organisms increase in frequency in a population. Natural selection can only occur when there is individual variation in a population, hence individual variation is central to natural selection. Although individual variation is a key concept for biology, the importance of considering the range of individual characteristics in populations, rather than just mean values, has only become apparent relatively recently (Bolnick et al., 2003, 2011; Cockrem, 2013b; Mimura et al., 2017).

When a population has a large amount of individual variation in a trait, then there will be animals that are suited to a wider range of situations than in a population where there is a small amount of variation in the trait. Populations with relatively high levels of individual variation are thus more likely to be able to survive in a range of environmental conditions than populations with low levels of individual variation. For example, if animals in one population all forage in the same area each day whereas animals in another population forage in several areas, then there is greater individual variation in foraging location in the second population. The second population will thus be less vulnerable than the first population to a shortage of food in one area. Maldonado-Chaparro et al. (2017) found that benefits of plasticity in body mass growth rate outweighed costs of plasticity in yellow-bellied marmots (*Marmota flaviventris*) in Colorado. Their modelling approach showed that individual variation in compensatory growth following harsh conditions decreased the probability of population extinction in unfavourable climate situations.

There can be disadvantages associated with individual variation, and Sih et al. (2004) noted that, in a simple view, natural selection should reduce variation and all individuals should be optimal in all environments. However, there can be multiple optimal behaviours for an environmental situation. An optimal behaviour for an animal in a situation involves a trade-off of costs and benefits. Individuals can differ in their

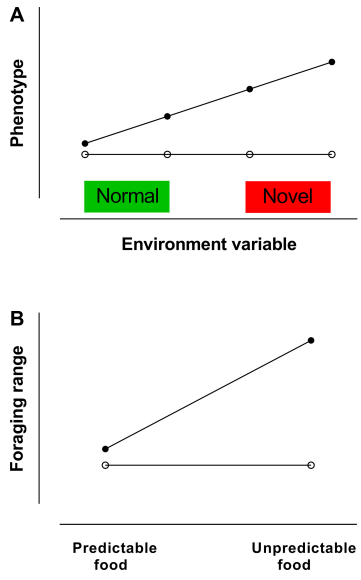

FIGURE 2
(A) Relationship between phenotype and level of an environmental variable for an animal with plasticity in responses to the variable in comparison with an animal that does not have phenotypic plasticity. **(B)** Example of relationships between foraging range and predictability of food supply for an animal with a reactive personality and an animal with a proactive personality. Reactive animals are more plastic than proactive animals and are more likely to respond to a change from a predictable to an unpredictable food situation by increasing their foraging range or changing their foraging locations.

trade-offs and hence exhibit different behaviours within the same environment, and there can be a range of behavioural types with similar fitness in a population (Sih et al., 2004). For example, specialists and generalists can coexist in a population (Moran, 1992). An extensive search of the literature for vertebrate and invertebrate species by Forsman and Wennersten (2016) found that greater genotypic and phenotypic individual variation in populations was indeed associated with lower vulnerability to environmental changes, smaller changes in population size, greater success and establishment in new areas, larger geographic ranges, and smaller risks of extinction. Experimental studies considered by Forsman and Wennersten (2016) generally showed that individual variation was more important under challenging conditions than benign conditions.

In addition to differences between animals in the level of a trait, there can also be differences between animals in the extent to which a trait can change when environmental conditions change. Differences between animals in the flexibility (plasticity) of traits can have important implications for the likelihood of success in coping with changing environments (Cockrem, 2013a), so in addition to individual variation in traits it is important to consider individual variation in plasticity of traits.

Individual variation in phenotypic plasticity

When an animal can change its behaviour or physiology in response to changes in its environment, the animal is showing phenotypic plasticity. Phenotypic plasticity is the capacity of a genotype to have different phenotypes in different environmental conditions (see **Figure 2A**), so phenotypic plasticity in a trait is the capacity of the trait to alter as environmental conditions change (see Valladares et al., 2006; Nussey et al., 2007). There is individual variation in the extent to which animals are flexible and plastic and can adjust to changing or unpredictable conditions (see **Table 3**). Results from a long-term study of a Dutch population of great tits by Nussey et al. (2005) are a good example of individual variation in plasticity in a trait. Birds with high plasticity in the timing of reproduction were able to advance their laying dates in years with warm spring temperatures and to delay their laying dates in years with cool spring temperatures. These birds were better able to match their reproductive timing with the peak in the availability of caterpillars to feed their offspring than birds with low plasticity in the timing of reproduction. Plasticity of laying dates was heritable, and there was a positive relationship between plasticity and fitness. Natural selection could therefore lead to changes in the composition of the population, in terms of laying date, with the proportion of birds with higher plasticity of laying date increasing and the population laying date being able to advance. The long-term success of the population will depend on the extent to which changes in the mean laying date are able to keep up with changes in caterpillar food availability that are occurring in response to warming spring temperatures due to climate change.

Populations that have relatively high individual variation in phenotypic plasticity are more likely to have animals that can adjust to changing environments than populations with low individual variation in plasticity. Variation within a

TABLE 3 Examples of individual variation in plasticity of behavioural and physiological responses of animals to changes in their environment.

Example	References
Individual variation in plasticity of behaviour	
Aggressiveness of male great tits	Araya-Ajoy and Dingemanse, 2017
Parental behaviour of the clown anemonefish (*Amphiprion percula*)	Barbasch and Buston, 2018
Undisturbed activity of male mosquitofish (*Gambusia affinis*)	Biro and Adriaenssens, 2013
Time spent in conspicuous positions in Namibian rock agamas	Carter et al., 2012
Migration date from New Zealand in bar-tailed godwits	Conklin et al., 2021
Foraging strategies in great tits	Coomes et al., 2022
Exploration behaviour in great tits	Dingemanse et al., 2012
Avoidance of risky habitat in American black bears (*Ursus americanus*)	Evans et al., 2019
Use of feeders at different air temperatures in blue tits	Herborn et al., 2014
Daily movement distance and diurnal activity in brown bears (*Ursus arctos*) in Sweden	Hertel et al., 2021
Boldness in three-spined sticklebacks	Jolles et al., 2019
Perceived predation danger for red knots (*Calidris canutus islandica*)	Mathot et al., 2011
Alternative reproductive tactics in male guppies (*Poecilia reticulata*)	Polverino et al., 2019
Egg-laying date of blue tits	Porlier et al., 2012
Individual variation in plasticity of glucocorticoid responses	
Faecal glucocorticoids in North American red squirrels (*Tamiasciurus hudsonicus*) at different densities	Guindre-Parker et al., 2019
Corticosterone responses of house sparrows (*Passer domesticus*) to food restriction	Lendvai et al., 2014

population in levels of phenotypic plasticity is thus related to the ability of populations to cope with changing environments associated with climate change, leading to the question of whether plasticity in relevant traits will be sufficient for a species to keep up with environmental changes (Wong and Candolin, 2014). Studies of individual variation in plasticity of behavioural and physiological responses of animals to changes in their environment (see **Table 3** for examples) are thus important for understanding responses of populations of animals to climate change.

Personality

Personality and responses to changes in the immediate environment

Patterns of daily behaviour and of responses to environmental changes are characteristics of individual animals that are remarkably consistent. Levels of flexibility in responses to stimuli from the environment, in other words, the amount of phenotypic plasticity in traits, are also characteristics of each animal. Terms for the individual differences in behaviour that are consistently expressed in different situations include behavioural syndrome (Dingemanse and Wright, 2020), temperament (Reale et al., 2007), and personality (Carere and Eens, 2005; Dingemanse et al., 2010; Stamps and Groothuis, 2010). Coping style, a related term defined as a coherent set of behavioural and physiological stress responses which is consistent over time (Koolhaas et al., 1999), refers to the capacity of individual animals to cope with environmental challenges.

Relationships between behavioural and physiological traits in animals have been explored in numerous studies. Reviews of such studies, for example McMahon et al. (2022), found reports of negative associations, positive associations, or no association between behavioural and physiological traits. This is not surprising, as the studies have been conducted in a wide range of captive and experimental situations, with varying methodologies, sample sizes, and numbers of behavioural variables measured. The studies have generally considered only a single physiological variable, and there is a need for studies incorporating several physiological processes at once to better characterise physiological profiles in relation to behavioural profiles (McMahon and Cavigelli, 2021; McMahon et al., 2022). Nonetheless, when results from many studies are considered together, regular patterns emerge for physiological profiles in relation to behavioural characteristics and it becomes apparent that behavioural and physiological responses are linked in animals. Personality can be defined as consistent individual behavioural and physiological responses of animals to changes in their immediate environment (Cockrem, 2007, 2013a,b). Coevolution of linked behavioural and physiological traits leads to consistency of these traits (Wolf and McNamara, 2012), and to the individual differences in suites of correlated behaviours expressed in different situations which are features of personalities (Carere and Eens, 2005). Personality is heritable, with approximately 52% of animal personality variation identified in a review of published studies attributable to additive genetic variation (Dochtermann et al., 2015).

While personality characteristics vary across a spectrum, animals can be classified into two broad groups. Animals that are highly responsive are said to have a reactive personality, and animals that have relatively smaller responses are said to have proactive personalities (Koolhaas et al., 1999; Sih et al., 2004; Cockrem, 2007). Proactive and reactive personality styles are equivalent to proactive and reactive coping styles. Personality styles can be apparent in animals in a wide range of situations. For example, proactive common brush tail possums (*Trichosurus vulpecula*) in Australia had a more diverse and higher quality diet than reactive possums (Herath et al., 2021), and reactive but not proactive Atlantic cod (*Gadus morhua*) reduced their home ranges as sea temperatures increased in a Norwegian fjord (Villegas-Ríos et al., 2018). Proposed characteristics of animals with proactive and reactive personalities are shown in **Table 4**. Animals with proactive personalities can be considered to have active responses

TABLE 4 Proposed characteristics of animals with proactive and reactive personalities [adapted from Cockrem (2007)].

Characteristic	Proactive	Reactive
Behavioural responses to threats	Fight-flight	Freeze-hide
Behavioural style	Aggressive and bold	Non-aggressive and cautious
Exploration	Fast and superficial	Slow and thorough
Behavioural flexibility	Rigid and routine-like	Flexible
Sensitivity to changes in the immediate environment	Less sensitive	More sensitive
Glucocorticoid responses	Relatively low	Relatively high
Success in constant compared with changing conditions	More successful in constant conditions	More successful in changing conditions
Success in predictable compared with unpredictable conditions	More successful in predictable conditions	More successful in unpredictable conditions
Plasticity of responses to changing environments	Relatively low	Relatively high
Ability to cope with environmental changes associated with climate change	Less able to cope	More able to cope

to immediate threats ("fight or flight" responses), to be relatively bold and aggressive, to be fast and superficial in their exploration of new situations, to be relatively rigid and routine-like in behaviour, and to have relatively low glucocorticoid responses to threatening stimuli [see Cockrem (2007) and Koolhaas et al. (2010)]. These animals have relatively low sensitivity to their immediate environment. Animals with reactive personalities can be considered to have passive responses to immediate threats (freeze-hide behaviour), have higher glucocorticoid responses to threats than proactive animals, and to be relatively slow and thorough to explore new situations [see Cockrem (2007) and Koolhaas et al. (2010)]. Reactive animals have relatively high sensitivity to their immediate environment and are more plastic (flexible) and more able to cope with changing circumstances than animals with proactive personalities (Geffroy et al., 2020). Differences between animals in cognition (mechanisms for the acquisition and processing of information from the environment) may contribute to the differences between personality styles in the sensitivity of animals to their immediate environment (Sih and Del Giudice, 2012).

Personality and coping with climate change

The environments in which animals have evolved are, at varying rates, changing due to climate change. Some populations of animals will be better able to cope with these environmental changes than other populations. There may be individuals in a population that can survive and breed in environmental conditions that differ from those experienced by the population in the past or can move to live in a new area. Alternatively, adaptation to novel environmental conditions can occur when individual animals have phenotypic plasticity in traits and can express phenotypes that enable the animals to survive. Natural selection for phenotypes that confer fitness in the changing situation (Fox et al., 2019) will lead to changes in the composition of the population. The survival of populations of animals will depend in part on the amount of individual variation in behavioural and physiological traits that contribute to fitness, together with the amount of individual variation in plasticity of these traits.

Plasticity can be adaptive (beneficial) or non-adaptive (Wilson, 1998). Adaptive plasticity can be acted upon by natural selection (Ghalambor et al., 2007), is favoured over fixed strategies when an environment is changing (Berrigan and Scheiner, 2004), and allows a genotype to have a broad tolerance to environmental conditions across multiple environments (Ghalambor et al., 2007). Plasticity that is non-adaptive and not subject to natural selection nonetheless enables animals to change in response to changing environmental conditions, and may "buy time" for a population to evolve and survive in environments that are changing due to climate change (Diamond and Martin, 2021). Plasticity may also have costs (Murren et al., 2015). Suggestions for potential costs of plasticity have included costs associated with cognition (Geffroy et al., 2020), energetic costs of the sensory and regulatory mechanisms of plasticity, and costs associated with the process of acquiring information about the environment which may be risky, involve energy for sampling, or reduce foraging or mating efficiency (DeWitt et al., 1998). Sih et al. (2004) noted that natural selection can favour the evolution of limited plasticity in situations where individuals have poor information about their environment, or if individuals avoid situations where limited plasticity would be a disadvantage. Conversely, Berrigan and Scheiner (2004) described how natural selection will favour plasticity over fixed strategies when the mean fitness of individuals with the plastic strategy exceeds that of individuals with the fixed strategy. Heterogeneity of the environment, in terms of changes in the environment with time or differences between areas in environmental conditions, was considered to be a necessary, but not sufficient, condition for plasticity to be favoured (Berrigan and Scheiner, 2004).

Traits that contribute to fitness are some of the traits that comprise the individual characteristics of animals known as personalities. It has been suggested that slow exploring reactive animals are less successful in stable environments than fast exploring proactive animals but are more successful in changeable environments (Dingemanse and Reale, 2005; Cockrem, 2007). For example, reactive animals more likely than proactive animals to respond to a change from a predictable to an unpredictable food situation by increasing their foraging range (see **Figure 2B**) or by changing their foraging locations. Animals with reactive personalities are likely to be better able to cope with environmental changes due to climate change than animals with proactive personalities, as suggested for birds by Cockrem (2013a). This suggestion is consistent with Sih (2013) who proposed that reactive, highly exploratory animals may be better able to cope with novel situations than proactive animals, and hence better able to cope with human-induced rapid environmental change (HIREC). In wild boars in Europe, slow explorers raised more offspring than fast explorers (Vetter et al., 2016), and fast exploring juvenile rabbits had lower survival than slow exploring rabbits (Rodel et al., 2015). Shy animals are more plastic than bold animals (Adriaenssens and Johnsson, 2011; Dammhahn and Almeling, 2012; Kareklas et al., 2016; Jolles et al., 2019). A study of free-living blue tits in Scotland showed that neophobic (reactive personality) birds changed their foraging behaviour at a feeder in response to changes in air temperature, whereas neophilic (proactive personality) birds did not change their foraging behaviour (Herborn et al., 2014). The reactive birds were more behaviourally flexible, had greater plasticity, and were better able to cope with a change in the environment than the proactive birds (Herborn et al., 2014).

If reactive animals that show plasticity in response to environmental change have higher fitness than other animals, then there is the potential for natural selection to lead to a change in the composition of the population so there is a greater proportion of animals with reactive personalities. Also, proactive animals may be more likely to move away from a habitat that changes than are reactive animals, so the remaining population would have a higher proportion of reactive animals than before the environmental change. The proportions of animals with different personalities may thus change over time in populations of animals that are experiencing environmental changes due to climate change, as suggested by Geffroy et al. (2020).

Glucocorticoids, personality, and fitness

Glucocorticoid hormones are secreted in reptiles, birds and mammals by the adrenal gland which is part of

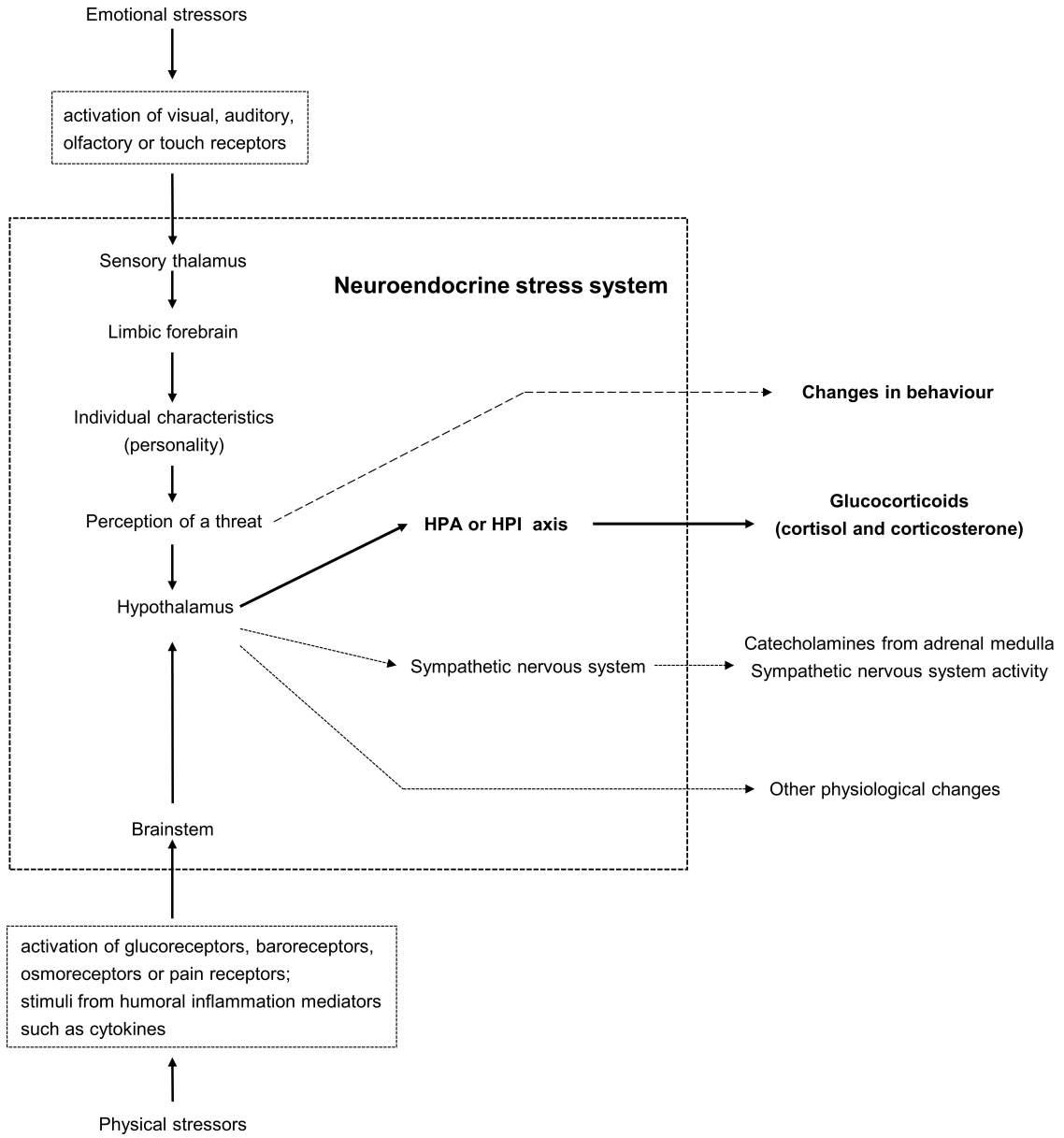

FIGURE 3
Schematic view of what is proposed to be called the neuroendocrine stress system. Responses of animals to threatening or potentially threatening changes in the external and internal environments arise from neural pathways and processes which can be called the neuroendocrine stress system, with stimuli that activate the system known as stressors.

the hypothalamo-pituitary-adrenal (HPA) axis. In fish and amphibians, glucocorticoids are secreted by the interrenal gland in the hypothalamo-pituitary-interrenal (HPI) axis. Cortisol is the predominant glucocorticoid in fish and most mammals, except rodents, and corticosterone is the predominant glucocorticoid in amphibians, reptiles, and birds.

Glucocorticoids are metabolic hormones with a primary function to increase blood glucose concentrations. These hormones have a wide range of other actions, including actions on behaviour, the immune system, the cardiovascular system, and the reproductive system (Sapolsky et al., 2000). Glucocorticoids are involved in responses of animals to stimuli from the environment that are perceived to be threatening (Cockrem, 2013b). They are often called stress hormones, almost always in the absence of a definition of stress, even though their primary roles are not in stress responses (MacDougall-Shackleton et al., 2019). Glucocorticoids are secreted into the blood, and glucocorticoid concentrations are measured in plasma and serum. Concentrations of glucocorticoids and of glucocorticoid metabolites are also measured in an increasingly wide variety of other sample types including saliva, urine, faeces, feathers, hair, and blubber. It should be noted that, despite widespread assumptions in the literature, concentrations measured in samples other than plasma, serum or saliva are not directly related to blood concentrations (Romero and Beattie, 2022). Glucocorticoid concentrations in blood samples collected from animals shortly after they are captured or restrained are often called baseline glucocorticoids, while changes in glucocorticoid concentrations after capture, restraint, or exposure to a stimulus thought to be threatening for an animal, are called glucocorticoid responses. However, glucocorticoid concentrations in blood increase rapidly when an animal is captured, restrained, or confined, measured concentrations may not accurately reflect concentrations in undisturbed animals, and the term baseline can be misleading.

Glucocorticoids are often measured in animals in relation to questions about responses of animals to changes in their environment. Glucocorticoid responses to stimuli from the environment are generally called stress responses. Stimuli that activate glucocorticoid responses act via receptors which send signals to what can be called the neuroendocrine stress system (see **Figure 3**). It is proposed that responses of animals to threatening or potentially threatening changes in the external and internal environments arise from neural pathways and processes which can be called the neuroendocrine stress system, with stimuli that activate the system known as stressors. This proposed system consists of neural pathways in the brain that convey information from the external and internal environments to the hypothalamus, neural structures that process this information, the HPA or HPI axis, and components of the sympathetic nervous system. Visual, auditory, olfactory and touch stimuli from the external environment that activate receptors in the eyes, ears, nose and skin and lead to activation of the neuroendocrine stress system can be called emotional stressors (Cockrem, 2007). Activation of these receptors

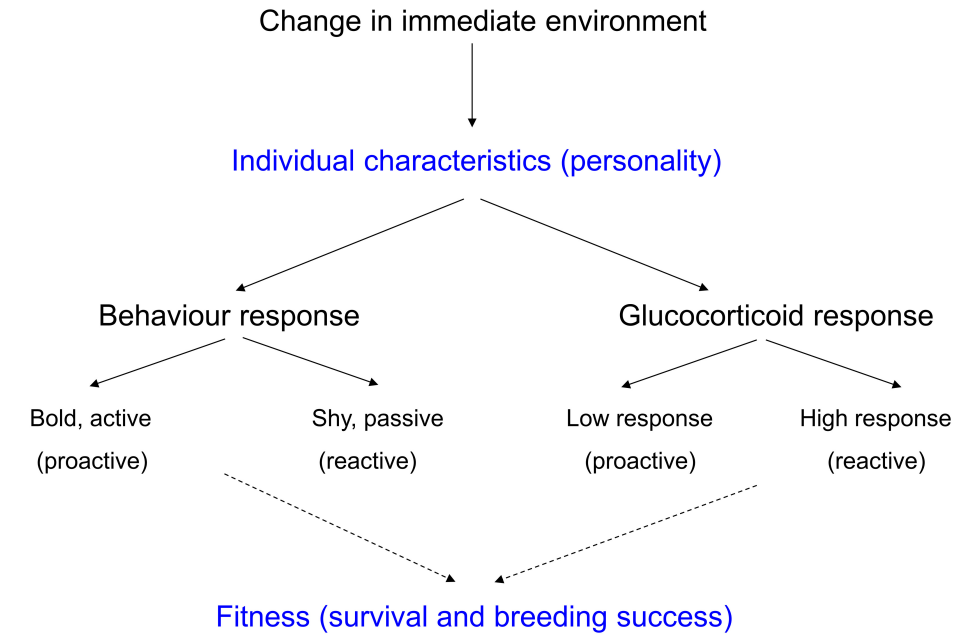

FIGURE 4
The influence of individual characteristics (personality) on behaviour responses, and glucocorticoid responses to changes in the immediate environment. These responses both influence fitness [based on Cockrem (2007, 2013a)].

generates signals that are processed by the limbic forebrain. It is suggested that individual characteristics known as personality influence whether a stimulus is perceived to be a threat and hence whether changes in behaviour, activity of the HPA or HPI axis, the sympathetic nervous system and other physiological pathways are activated (Cockrem, 2007). Stimuli that activate the neuroendocrine stress system without processing of the signals by the limbic forebrain can be called physical stressors (Cockrem, 2007). These stimuli can come from the internal environment via activation of glucoreceptors, baroreceptors and osmoreceptors, from the external or internal environments via the activation of thermoreceptors or pain receptors, and from humoral signals of inflammation such as cytokines.

A glucocorticoid response is a response to a stimulus from the immediate environment that is perceived as a threat, usually a visual stimulus. Glucocorticoid responses can be detected as increased concentrations of glucocorticoids in the blood within several minutes of a stimulus beginning (Romero and Reed, 2005). The duration of a response depends on the extent to which the stimulus is perceived to be a threat and on the duration of the stimulus. There is marked variation between individual animals in their glucocorticoid responses (Cockrem, 2013b). Some animals consistently have little or no response to a stimulus that initiates a large response in other animals, so a stimulus that is perceived as very threatening by one animal can be perceived quite differently by another animal. Higher glucocorticoid responses in some animals compared with others reflect an increased biological sensitivity to context in the animals with higher glucocorticoid responses (Boyce and Ellis, 2005). In other words, some animals are more aware of, and more sensitive to stimuli from their immediate environment than other animals. It has been suggested that the size of a glucocorticoid response of an animal is determined by individual characteristics of the animal which can be called personality (see **Figure 4**; Cockrem, 2007, 2013a).

It has been assumed that fitness in animals [the ability of animals to survive and breed (Orr, 2009)] is determined by baseline glucocorticoids or by glucocorticoid responses [see the Cort-Fitness Hypothesis; (Bonier et al., 2009)]. However, fitness in animals is not determined by glucocorticoid hormones alone, and differences between animals in fitness are not a direct consequence of differences between animals in baseline glucocorticoids or the size of the glucocorticoid responses. Relationships between glucocorticoids, personality and fitness are usually overlooked in the literature on the significance of glucocorticoid responses in relation to fitness in animals. Survival and breeding success depend on the behaviour and physiology of animals, not just on glucocorticoids, and is not surprising that there is no clear support for any of the three main hypotheses about glucocorticoids and fitness (Breuner and Berk, 2019; Romero and Gormally, 2019).

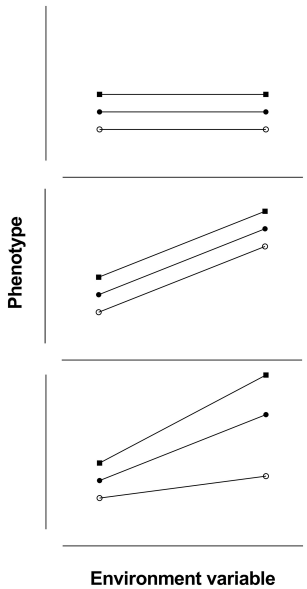

FIGURE 5
Reaction norms for individual animals with no phenotypic plasticity (reaction norms parallel with slopes equal to zero; upper panel), the same degree of phenotypic plasticity (reaction norms parallel with slopes not equal to zero; middle panel), and individual variation in plasticity (reaction norms not parallel with slopes not equal to zero; lower panel).

Reaction norms and responses of animals to changing environments

Reaction norms

The persistence of populations of animals in changing environments in coming decades will depend, in part, on

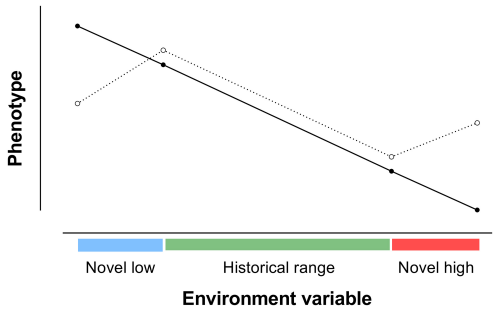

FIGURE 6
Reaction norms for an animal with the same slope of reaction norm in historical and novel environmental conditions (— ● —) and for an animal with different slopes in novel conditions in comparison with an historical range of conditions (···○···). The reaction norm for the second animal in historical conditions did not predict the reaction norm in novel conditions [adapted from Ghalambor et al. (2007)].

the abilities of individual animals to show plasticity in behavioural and physiological characteristics that affect the likelihood of successful breeding and survival. Understanding of relationships between individual characteristics and environmental variables requires the description and quantification of the relationships. The reaction norm approach is widely used in studies of plants and animals for situations where a phenotype varies in relation to an environment variable. The term reaction norm was first used in its current sense by Schmalhausen et al. (1949), and began to be used regularly in discussions of phenotypic plasticity from the 1980s [e.g., Stearns (1989)]. A reaction norm can be defined as "the set of phenotypes that can be produced by an individual genotype that is exposed to different environmental conditions" (Schlichting and Pigliucci, 1998). Other definitions have included "the way that an individual's phenotype varies across environments" (Ghalambor et al., 2007) and "a function describing the change in a genotype's phenotype across an environmental gradient" (Nussey et al., 2007).

Reaction norms are commonly depicted on graphs with a trait on the *y*-axis and an environment variable on the *x*-axis. The environment variable may be continuous, for example ambient temperature, or discrete, for example two situations in which a trait was measured. The slope of a reaction norm for an individual animal is a measure of phenotypic plasticity. **Figure 2A** shows reaction norms for an animal with the same phenotype at different levels of an environment variable (slope equals zero, no plasticity in the phenotypic trait), and for an animal whose phenotype changed at different levels of the environment variable (positive slope, plasticity in the trait). **Figure 2B** shows reaction norms for foraging range in relation to levels of food availability.

Individual animals can have phenotypes that do not change in different environment conditions (parallel reaction norms with slopes that equal zero, no plasticity), phenotypes that change at the same rate (parallel reaction norms with same positive or negative slopes, same plasticity), or phenotypes that change at different rates (non-parallel reaction norms with different slopes, individual variation in plasticity). These three situations are shown in **Figure 5**.

The slope of a reaction norm, the rate of change in a phenotype in relation to changes in an environmental variable, can be used to predict the phenotype of an animal outside the measured range of the environmental variable. However, in some animals the slope at levels of the environment variable outside the historical range may differ from the slope measured within the historical range (**Figure 6**). Reaction norm measurements are thus needed both in free-living animals exposed to natural environment conditions, and in experimental situations where levels of an environment variable can be lower or higher than the historical range of levels to which the species has been exposed naturally.

The use of reaction norms for understanding how individual animals and populations may cope with climate change

Recognition of the importance of individual variation in plasticity of responses of animals to changing environments, and the use of reaction norms to identify this variation, is relatively recent. Dingemanse et al. (2010) described behavioural reaction norms for identification of individual plasticity in relation to animal personality. Cockrem (2013a) showed how reaction norms can be used to quantify plasticity and individual variation in plasticity of glucocorticoid responses and suggested that reaction norms for corticosterone responses in birds could be used to predict how birds could cope with environmental changes due to climate change. Brommer (2013) reviewed studies that showed individual variation in plasticity of traits in animals, Valladares et al. (2014) modelled reaction norms to show how individual variation in plasticity of response to temperature could be used to predict changes in species distribution, and Taff and Vitousek (2016) described how reaction norms could be used to study individual variation in endocrine flexibility. Some reaction norm studies that reported individual variation in plasticity for free living animals and for animals in experimental situations are listed in **Table 5**.

TABLE 5 Examples of reaction norm studies that showed individual variation in slopes (individual variation in plasticity).

Example	References
Baseline corticosterone in house sparrows subjected to experimental changes in temperature, wind speed, and food predictability	Baldan et al., 2021
Resting metabolic rate at two environmental temperatures in alpine newts (*Ichthyosaura alpestris*)	Baškiera and Gvoždík, 2022
Behaviour (time spent conspicuous) in Namibian rock agamas in different seasons	Carter et al., 2012
Parturition date in viviparous skink (*Niveoscincus ocellatus*) in relation to environmental temperature	Cunningham et al., 2020
Experimental test of exploration behaviour in great tits	Dingemanse et al., 2012
Faecal glucocorticoids in North American red squirrels (*Tamiasciurus hudsonicus*) at different densities	Guindre-Parker et al., 2019
Baseline corticosterone in house sparrows subjected to experimental changes in food availability	Lendvai et al., 2014
Timing of egg laying in great tits in the Netherlands	Nussey et al., 2005
Insulin-like growth factor-1 in bearded reedlings (*Panurus biarmicus*) at two experimental levels of food availability	Toth et al., 2022

The reaction norm approach enables identification of individual variation in plasticity of responses of animals to changing environments. Reaction norm graphs for individual animals, such as those in **Figure 5**, show the extent to which animals in a population have phenotypic plasticity for a trait in different environmental conditions. If the slopes of reaction norms are low, then the animals have low plasticity and the capabilities of animals in the population to change a trait in response to changing environmental conditions are low. Conversely, if reaction norm slopes are high, then animals have relatively high capabilities to change as environmental conditions change. Importantly, when the slopes of reaction norms for individual animals are not parallel, then the reaction norm graph shows individual variation in plasticity and hence the extent to which individual animals differ in their responses to changing conditions.

Phenotypic plasticity, the capacity of a trait to have different phenotypes in different environmental conditions, is important for individual animals and hence for populations to be able to cope with changes in their environments associated with climate change (Sih et al., 2011; Wong and Candolin, 2014; Fox et al., 2019). Individual variation in plasticity is also important (Kelly, 2019). Populations with relatively high individual variation in plasticity of reaction norms, will have more animals that can adjust to a new situation than populations with little variation in plasticity. The populations with high variation in plasticity are more likely to persist as environments change due to climate change than populations with low individual variation in plasticity. Studies that use reaction norms to identify phenotypic plasticity and individual variation in plasticity in responses of animals to changes in environment variables can inform conservation policy (Cooke et al., 2021), and will be valuable for understanding how populations of animals may cope with climate change.

Conclusion

The Sixth Assessment of the Intergovernmental Panel on Climate Change, published in 2021 and 2022, describes negative effects of climate change on animals occurring more rapidly and on a larger scale than previously appreciated. Animal species are increasingly experiencing adverse weather conditions that differ in frequency and intensity from the conditions in which the species evolved. Individual variation in behavioural and physiological responses of animals to stimuli from the environment is ubiquitous across all species. There is also individual variation in the extent to which animals are flexible and plastic and can adjust to changing or unpredictable conditions. Populations that have relatively high individual variation in plasticity are more likely to have some individuals that can cope with climate change than populations with low individual variation in plasticity. Reaction norms, which show phenotypic plasticity in traits, are useful for identifying this plasticity. Future studies of individual variation in plasticity of responses to changing environments will help understanding of how populations of animals may be able to cope with climate change.

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Conflict of interest

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

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