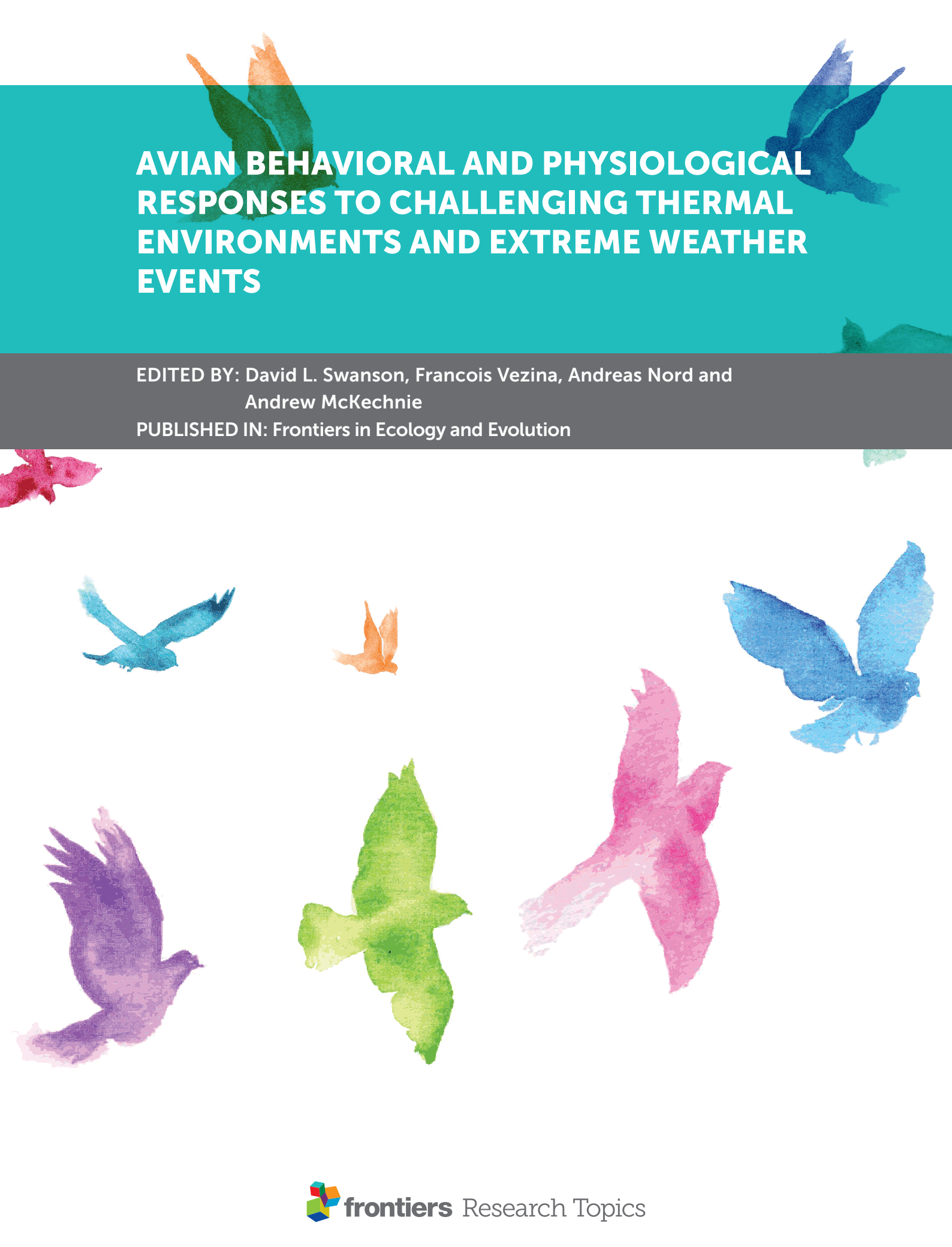


The background of the cover features a teal header and a white footer, with watercolor illustrations of birds in flight. In the teal header, there are two birds: one in shades of green and orange on the left, and one in shades of blue and purple on the right. In the white footer, there are five birds in various colors: a blue bird on the left, a small orange bird in the center, a large blue bird on the right, a purple bird on the bottom left, and a green bird on the bottom center. A pink bird is also visible on the bottom right.

AVIAN BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO CHALLENGING THERMAL ENVIRONMENTS AND EXTREME WEATHER EVENTS

EDITED BY: David L. Swanson, Francois Vezina, Andreas Nord and
Andrew McKechnie

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AVIAN BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO CHALLENGING THERMAL ENVIRONMENTS AND EXTREME WEATHER EVENTS

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Editorial: Avian behavioral and physiological responses to challenging thermal environments and extreme weather events

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Editorial on the Research Topic

Avian behavioral and physiological responses to challenging thermal environments and extreme weather events

Introduction

Birds occupy habitats ranging from Antarctic ice shelves to tropical deserts and lowland rainforests, so are exposed to the full range of climates on Earth (Dawson and O'Connor, 1996). Cold, hot or spatially and temporally variable thermal conditions can present significant thermoregulatory challenges to birds, which typically must maintain body temperatures (T_b) within narrow physiological limits (McKechnie, 2022). Such challenges may occur throughout the year (Parr et al., 2019) and in all life stages (DuRant et al., 2012; Nord and Giroud, 2020), so adjustments to these conditions are required to maintain fitness and, ultimately, stable populations. Here, we broadly define a challenging thermal environment as one requiring physiological acclimation or behavioral adjustments that modify rates of thermogenesis or heat loss to maintain long-term ecological function.

Avian abilities to respond physiologically to extreme temperatures are defined by capacities for heat production or dissipation (Swanson, 2010; McKechnie et al., 2021a). Behavioral responses to environmental temperature reduce the magnitude of physiological adjustments, although potentially with opportunity costs (Cunningham et al., 2021). It is this combination of behavioral and physiological responses at multiple levels of organization that determines the survival probability of birds in thermally challenging situations (e.g., Albright et al., 2017; Petit et al., 2017). Moreover, thermal conditions experienced during reproduction can affect parental investment and nestling development, with potentially long-term consequences (Nord and Giroud, 2020; van de Ven et al., 2020; Broggi et al., 2022). Our knowledge of response mechanisms, their time courses, and their impacts on fitness, however, remains incomplete. Behavioral and physiological responses of birds to extreme and/or seasonally variable climates have been a research focus for decades (Chaffee and Roberts, 1971; Dawson et al., 1983), but recent methodological and analytical advances for studies of physiology and behavior have produced novel findings regarding patterns and mechanisms of avian adjustments to challenging thermal environments (e.g., McCafferty et al., 2015; Cheviron and Swanson, 2017; McKechnie et al., 2021a).

Avian responses to heat and aridity

Physiological and behavioral responses permit the maintenance of sublethal T_b under hot conditions, but water is required for evaporative cooling, so interactions between temperature and water availability are important considerations for thermoregulation in the heat (Conradie et al., 2020). Large birds have greater thermal inertia and lower surface area to volume ratios than small species, so body mass may impact the magnitude of heat tolerance responses (McKechnie et al., 2021a), but this has been little studied. Czenze et al. found that heat tolerance, maximum T_b , and evaporative cooling capacities in three larger-bodied South African non-passerines approximated those in other non-passerines and exceeded capacities in passerines (McKechnie et al., 2021a). Sabat et al. tested a new method to estimate metabolic and pre-formed water contributions to the body water pool and detected isotopic differences under cold temperatures and between species using freshwater and saltwater resources, thereby validating the method for future studies of water balance. Navarette et al. experimentally manipulated water availability in rufous-collared sparrows (*Zonotrichia capensis*) and identified trade-offs involving water restriction-induced increases in basal metabolic rate (BMR) and erythrocyte oxidative enzyme activities at the expense of skeletal muscle oxidative damage. Sharpe et al. documented reduced foraging and increased use of thermally buffered microhabitats by Jacky Winters (*Microeca fascians*)

during hot weather; nevertheless, 29% of the study population died when air temperature reached 49°C, demonstrating limits to physiological and behavioral capacities for responding to extreme heat events.

Avian responses to heat during reproduction

The heat dissipation limits hypothesis (HDLH) posits that the capacity to dissipate heat loads acquired during sustained activities, such as breeding, limits performance and may negatively affect reproductive output and fitness (Speakman and Król, 2010). Several studies of free-living birds support the HDLH, even in comparatively cool habitats (Andreasson et al., 2020). Zagkle et al. found support for the HDLH by manipulating heat loss while increasing foraging costs in zebra finches (*Taeniopygia guttata*), documenting negative effects on reproduction under warm temperatures that were buffered by experimentally increased heat loss. Increasing temperatures over an 11-year study period were strongly negatively correlated with reproductive output in southern yellow-billed hornbills (*Tockus leucomelas*) (Pattinson et al.), suggesting that, if current warming trends continue, reproductive capacity will be sufficiently compromised to result in imminent nesting failure for this population. Pipoly et al. demonstrated that negative effects of high temperatures on nestling growth and survival were stronger in forest than urban populations of great tits (*Parus major*), suggesting that urban nestlings are less vulnerable to heat. Udino and Mariette experimentally documented that parental heat calls during the late *in ovo* period resulted in panting at lower temperatures, reduced panting at high temperatures, and higher activity at warm temperatures when the offspring had reached adulthood, highlighting the priming effects of early life conditions on later thermoregulatory patterns.

Avian responses to cold

Metabolic flexibility allows birds to match metabolic rates to environmental conditions (Swanson, 2010). Underlying mechanisms of metabolic flexibility include adjustments in muscle size (Swanson and Vézina, 2015; Swanson et al., 2022) and cellular aerobic and fat catabolism capacities (Swanson, 2010), but the contribution of other metabolic pathways to this flexibility is poorly known (Stager et al., 2015; Cheviron and Swanson, 2017). Wone and Swanson used integrated metabolomics/transcriptomics analyses to document seasonal changes in amino acid, lipid- and cellular metabolism pathways in two passerine birds and identified a potential role for nicotinamide-adenine-nucleotide derivatives in regulating cellular metabolism. In addition to heat production mechanisms, energy conservation strategies, including torpor

(Ruf and Geiser, 2015; Geiser, 2021) and ventilatory/respiratory adjustments (Arens and Cooper, 2005), can contribute to avian cold tolerance. Bech and Mariussen detected winter increases in BMR and the respiratory frequency/tidal volume ratio in great tits, allowing energy savings by reducing respiratory energetic costs and evaporative water losses. Aharon-Rotman et al. showed that winter-acclimatized eastern yellow robins (*Eopsaltria australis*) regularly entered torpor, expanding documentation of torpor use in the comparatively poorly studied passerine taxon.

Conclusions

As demonstrated by the studies in this Research Topic, behavioral and physiological flexibility can buffer temperature impacts on birds. In addition to changes in average temperatures, however, global change is predicted to increase climate variability, with more frequent extreme events for many locations (Jentsch et al., 2007; Wallace et al., 2014; Cohen et al., 2018). Increasing extreme summer maximum temperatures and more variable winter temperatures can have negative consequences for birds, including mass mortality events (McKechnie and Wolf, 2010; McKechnie et al., 2021b), phenotype-environment mismatches (Boyles et al., 2011; Jimenez et al., 2020; Vézina et al., 2020; Ruuskanen et al., 2021), reduced reproductive capacities (Carroll et al., 2018; Nord and Nilsson, 2019; van de Ven et al., 2020), and altered offspring physiology and behavior (Mariette and Buchanan, 2016; Mariette, 2020). Future research incorporating not only behavior and physiology, but also flexibility in these traits and their thermal reaction norms, into population and distribution models will be critical to understand impacts of climate change on avian biodiversity.

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Triple Oxygen Isotope Measurements ($\Delta^{17}\text{O}$) of Body Water Reflect Water Intake, Metabolism, and $\delta^{18}\text{O}$ of Ingested Water in Passerines

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Understanding physiological traits and ecological conditions that influence a species reliance on metabolic water is critical to creating accurate physiological models that can assess their ability to adapt to environmental perturbations (e.g., drought) that impact water availability. However, relatively few studies have examined variation in the sources of water animals use to maintain water balance, and even fewer have focused on the role of metabolic water. A key reason is methodological limitations. Here, we applied a new method that measures the triple oxygen isotopic composition of a single blood sample to estimate the contribution of metabolic water to the body water pool of three passerine species. This approach relies on $\Delta^{17}\text{O}$, defined as the residual from the tight linear correlation that naturally exists between $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ values. Importantly, $\Delta^{17}\text{O}$ is relatively insensitive to key fractionation processes, such as Rayleigh distillation in the water cycle that have hindered previous isotope-based assessments of animal water balance. We evaluated the effects of changes in metabolic rate and water intake on $\Delta^{17}\text{O}$ values of captive rufous-collared sparrows (*Zonotrichia capensis*) and two invertivorous passerine species in the genus *Cinclodes* from the field. As predicted, colder acclimation temperatures induced increases in metabolic rate, decreases in water intake, and increases in the contribution of metabolic water to the body water pool of *Z. capensis*, causing a consistent change in $\Delta^{17}\text{O}$. Measurement of $\Delta^{17}\text{O}$ also provides an estimate of the $\delta^{18}\text{O}$ composition of ingested pre-formed (drinking/food) water. Estimated $\delta^{18}\text{O}$ values of drinking/food water for captive *Z. capensis* were $\sim -11\text{‰}$, which is consistent with that of tap water in Santiago, Chile. In contrast, $\delta^{18}\text{O}$ values of drinking/food water ingested by wild-caught *Cinclodes* were similar to that of seawater, which is consistent with their reliance on marine resources. Our results confirm the utility of this method for quantifying the relative

contribution of metabolic versus pre-formed drinking/food water to the body water pool in birds.

Keywords: birds, $\Delta^{17}\text{O}$, evaporative water, metabolic rate, metabolic water, stable isotopes

INTRODUCTION

Understanding the physiological mechanisms that species use to maintain water balance is becoming more relevant as increases in temperature and drought frequency represent significant ecological shifts that are affecting the behavior, distribution, and abundance of animals (McCarty, 2001; Albright et al., 2010; Şekercioğlu et al., 2012; IPCC, 2013; Remeš and Harmáčková, 2018). Because of their diurnal habits and high mass-specific metabolic rates, birds are particularly susceptible to increases in temperature and aridity (Riddell et al., 2021), so better understanding the environmental factors that influence their water balance is an important topic of research. Recent studies highlight that warm temperatures and reduced availability of fresh water impact key aspects of avian physiology, such as energy expenditure, body mass, thermal tolerance/conductance, and evaporative water loss (Carmi et al., 1993; Sabat et al., 2006a, 2009; Barceló et al., 2009; Gerson and Guglielmo, 2011; Smith et al., 2017; McWhorter et al., 2018). In addition to inducing physiological changes, thermal and water stress can also affect behavior, species distribution, and fitness. For instance, using a combination of physiological data, mechanistically informed models and climatic data predicted that the proportion of the ranges of the distribution of avian species with risk of lethal dehydration during heat waves will dramatically increase under future climate scenarios (Albright et al., 2017).

Most metabolic and functional processes of terrestrial animals are sensitive to water balance, where the steady state homeostatic water budget (intake = loss) assumes a constant amount of total body water:

$$\text{WI}_D + \text{WI}_{PF} + \text{WI}_M = \text{WL}_C + \text{WL}_R + \text{WL}_U + \text{WL}_{FC} \quad (1)$$

where WI_D is drinking water; WI_{PF} is (preformed) water in food; WI_M is metabolic water formed in the aerobic metabolism of dietary macromolecules; WL_C is cutaneous loss of water thorough the skin; WL_R is the loss of water through respiratory surfaces; and WL_U and WL_{FC} are the loss of water through urine and feces, respectively. For most birds, the total evaporative water loss (TEWL), which is the sum of cutaneous and respiratory losses ($\text{WL}_C + \text{WL}_R$), accounts for between 50 and 80% of total losses depending on hydration conditions, while urine and feces ($\text{WL}_U + \text{WL}_{FC}$) account for only 15–30% of total water losses (Goldstein and Braun, 1986; MacMillen, 1990; Goldstein and Skadhauge, 2000). The relative contribution of WI_D , WI_{PF} , and WI_M to an animal's total water budget depends on environmental conditions (e.g., temperature, humidity, and water intake), the rate and macromolecular substrate (protein, carbohydrates, and/or lipids) oxidation, and behavioral attributes (e.g., diurnal versus nocturnal activity). Ultimately, an organism's water balance is a function of the interplay between the physical

environment, the physiological and/or behavioral mechanisms for conserving water (minimizing losses), and the production of metabolic water which is directly linked to metabolic rate (Bartholomew and Cade, 1963; MacMillen, 1990; Gerson and Guglielmo, 2011; Rutkowska et al., 2016; Albright et al., 2017).

The contribution of metabolic water to the body water pool is highly variable among birds (MacMillen, 1990; Williams et al., 1993; Sabat et al., 2006a). Because TEWL accounts for the largest proportion of water lost by birds (McKechnie and Wolf, 2004), the WI_M/TEWL ratio is especially informative: As this ratio increases toward unity, birds rely more heavily on metabolic water to maintain water balance. Importantly, the physiological traits and ecological conditions that constrain this ratio by either favoring or limiting reliance on metabolic water and thus potential independence from environmental water remain largely unknown (Bartholomew and Cade, 1963; MacMillen, 1990). For instance, what role does metabolic water production (WI_M) play in maintaining water balance during physiological challenges related to thermoregulation? Does reliance on different water sources vary with thermoregulatory demands? Understanding these mechanisms is critical to creating accurate physiological models that can assess the ability of animals to adapt to potential threats caused by anthropogenic and natural environmental perturbations, especially increases in ambient temperature and drought frequency predicted for many regions over the next century (Walther et al., 2002; Vale and Brito, 2015; Iknayan and Beissinger, 2018). These perturbations are especially relevant for birds from the order Passeriformes because most species are diurnal and have small body masses, high body temperatures, and high mass-specific metabolic rates that make them particularly susceptible to thermal and dehydration stress (McKechnie and Wolf, 2010; Albright et al., 2017).

Several studies have shown that in comparison with their counterparts that occur in more mesic environments, birds inhabiting aridland ecosystems exhibit physiological adjustments to prevent water loss (Casotti and Braun, 2000; Williams and Tieleman, 2005; McKechnie et al., 2016; Gerson et al., 2019). However, few avian studies have examined variation in the use of potential sources of water to maintain water balance (Navarro et al., 2018; Smit et al., 2019), and even fewer studies have focused on the role of metabolic water in the body water budgets of birds (Williams, 2001; Giulivi and Ramsey, 2015). One of the primary limitations is the inability to assess the contribution of metabolic water to the body water pool without the use of injected tracers (e.g., $^2\text{H}_2^{18}\text{O}$) that require multiple captures of the same individual over short periods of time (Butler et al. 2004). Recently, Whiteman et al. (2019) proposed a new method for estimating the contribution of metabolic water to the body water pool based on the measurement of $\Delta^{17}\text{O}$, which is the positive or negative deviation from the

tight linear correlation that naturally exists between $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ values (Sharp et al., 2018; Whiteman et al., 2019). As shown in equation (1), body water inputs primarily include drinking water (W_D) and food water (W_{PF}), both of which are ultimately derived from meteoric water (i.e., precipitation), and metabolic water (W_{IM}). Metabolic water (W_{IM}) is assumed to have a $\Delta^{17}\text{O}$ value of -0.44‰ reflecting that of inhaled atmospheric oxygen (Liang et al., 2006; Wostbrock et al., 2020). In contrast, the $\Delta^{17}\text{O}$ value of W_D and W_{PF} is that of meteoric water, which is approximately $+0.03\text{‰}$ regardless of the source (Li et al., 2015; Sharp et al., 2018; Passey and Ji, 2019). $\Delta^{17}\text{O}$ values of meteoric water have this consistent value because mass-dependent fractionation associated with evaporation and condensation affects all three oxygen isotopes in a similar and predictable fashion (Sharp et al., 2018). By extension, evaporation during physiological processes (e.g., gular fluttering) should have minimal effect on $\Delta^{17}\text{O}$ values of animal body water.

A linear mixing model can be used to calculate the proportional contribution from drinking/food versus metabolic water (Whiteman et al., 2019). Because drinking/food water and metabolic water together provide 80–99% of the body water of most animals (Bryant and Froelich, 1995; Kohn, 1996), we can ignore the remaining minor contribution (1–20%) from water formed in condensation reactions from the bound oxygen in dietary macromolecules, and model bird body water ($\Delta^{17}\text{O}_{BW}$) as:

$$\Delta^{17}\text{O}_{BW} = F_M \times (-0.44\text{‰}) + (1 - F_M) \times (0.030\text{‰}) \quad (2)$$

where F_M represents the fractional contribution to body water from metabolic water, and $(1 - F_M)$ represents the contribution from pre-formed (drinking/food) water. Whiteman et al. (2019) showed that this equation accurately predicted relative changes in $\Delta^{17}\text{O}$ values of captive deer mice based on their metabolic rate and drinking water intake and that $\Delta^{17}\text{O}$ measurements in wild mammals appeared to reflect expected variation in relative mass-specific rates of metabolism and water intake. Continued research is required to assess additional potential predictors for this model, such as evaporation-driven variation in $\Delta^{17}\text{O}$ (as described above), and trophic enrichment in which food water is increasingly influenced by prey metabolic water for higher-trophic level consumers. Another important need is applying this simplified model (equation 2) to non-mammalian taxa.

Assuming a fixed $\Delta^{17}\text{O}$ value of 0.03‰ for meteoric water is reasonable, but the potential for variation should be noted. Regarding precipitation, patterns emerge at high and low values of $\delta^{18}\text{O}$: $\Delta^{17}\text{O}$ is closer to 0.01‰ if $\delta^{18}\text{O}$ is above -10‰ and closer to 0.04‰ if $\delta^{18}\text{O}$ is below -25‰ (Passey and Levin, 2021). Unique environmental conditions can alter $\Delta^{17}\text{O}$ more dramatically: For example, if $\sim 90\%$ of a closed water body evaporates into air with very low relative humidity, the $\Delta^{17}\text{O}$ of the remaining water may fall as low as -0.20‰ (Passey and Levin, 2021); evaporation of water from plants in very dry conditions can have a similar effect (Landais et al., 2006; Li et al., 2017). However, many environmental sources of meteoric water are not subject to the conditions required to cause such variation in meteoric water $\Delta^{17}\text{O}$. In addition, while

deviation from 0.03‰ represents important information for hydrological and geochemical studies, the mean $\Delta^{17}\text{O}$ value for meteoric water (0.03‰) is very distinct from the biological signal of metabolic water (-0.44‰).

Here, we explore $\Delta^{17}\text{O}$ in birds. We consider the responses of metabolic rate, TEWL, and the contribution of metabolic water to the body water pool in a widely distributed passerine, the rufous-collared sparrow (*Zonotrichia capensis*), with captive experiments of 15-day exposure to cold (15°C) followed by warm (30°C) environmental conditions. We hypothesize that birds acclimated to the cold conditions will have relatively higher resting metabolic rates (RMRs) but will consume less drinking water than when acclimated to warm conditions. We predict that these responses will yield a change in body water $\Delta^{17}\text{O}$ values that reflect a net increase in the contribution of metabolic water to the body water pool during cold conditions. Unlike previous applications of oxygen isotopes that have focused exclusively on $\delta^{18}\text{O}$, our triple isotope approach is much less sensitive to evaporative ^{18}O -enrichment of body water nor does it require isotopic characterization of all potential water sources. A novel contribution of our study is the application of a new analytical method that estimates the relative contributions drinking and food water ($W_D + W_{PF}$) vs. metabolic water (W_{IM}) to the body water pool (Whiteman et al., 2019) based on the analysis of a single blood plasma sample. In addition to using this approach to study water balance in captive sparrows, we also report data on the contribution of metabolic water to the body water pool in two species of wild-caught songbirds in the genus *Cinclodes* (*Cinclodes oustaleti* and the *Cinclodes nigrofumosus*), a coastal group of invertivorous passerines that vary in their ability to use marine resources. Our approach combines phenomenological data collected from the field with results from laboratory experiments designed to identify the physiological mechanisms that constrain how animals respond to environmental conditions (Khaliq et al., 2014). The results improve our understanding of the physiological responses to climate change and the ultimate threats to species' persistence.

MATERIALS AND METHODS

Sample Collection

Our captive model species was the omnivorous rufous-collared sparrow, which is widely distributed across a range of habitats in western and southern South America (Araya et al., 2005). We captured 10 individuals using mist nets in the Quebrada de la Plata ($33^\circ 31'\text{S}$, $70^\circ 5'0''\text{W}$, $\sim 500\text{m}$ elevation) in central Chile, a locality with a Mediterranean climate. Following capture, we transported birds to the laboratory for a 2-day habituation period at 22°C . The birds were maintained in individual cages ($50 \times 50 \times 50\text{cm}$) and were fed *ad libitum* with dried birdseed and water. Water was offered in inverted 100 ml graduated plastic tubes that allowed birds to eat and drink in a small ($\sim 1\text{cm}^2$) container at the bottom of the tube. After the habituation period, birds were maintained at $15 \pm 0.5^\circ\text{C}$ for 15 days, and then at $30 \pm 0.5^\circ\text{C}$ for another 15 days (12:12 light:dark

photoperiod). This acclimation period was long enough to ensure complete turnover of the body water pool for a 20–30 g passerine (Bartholomew and Cade, 1963; Smit and Mckechnie, 2015). After each cold or warm acclimation period, we collected samples of blood (50–100 µl) in the morning (09:00–11:00 h) from the humeral vein using hematocrit tubes with anticoagulant (heparine). Blood samples were then centrifuged at 10,000 rpm (relative centrifugal force = 9,250) for 5 min during which plasma was separated from red blood cells, and then, plasma was frozen at -80°C until isotope analysis. Water intake rates were measured with the inverted graduated plastic tubes and corrected for evaporation by using control tubes located outside each experimental cage.

Wild *C. oustaleti* and *C. nigrofumosus* were collected using mist nets in the austral winter (June 2018) at Los Molles ($32^{\circ}14'22''\text{S}$ $71^{\circ}30'54''\text{W}$) on the central coast of Chile. Blood samples were obtained with heparinized microcapillary tubes from the humeral vein immediately after capture. Blood was centrifuged at 10,000 rpm for 10 min and the plasma was separated from red blood cells and stored at -80°C until isotope analysis.

Metabolic Water Analysis

To measure $\Delta^{17}\text{O}$, we cryogenically distilled water from 1 to 2 µl blood plasma samples in a vacuum line, then reacted it with BrF_5 at $\sim 300^{\circ}\text{C}$ for 5–10 min, quantitatively converting H_2O to O_2 and other gasses. These other gasses were removed *via* liquid nitrogen traps and the O_2 was further purified by passing it through a zeolite molecular sieve and a gas chromatography column. O_2 was then analyzed on a dual-inlet Thermo Scientific 253 isotope ratio mass spectrometer (Bremen, Germany) at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). The measured values of $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ were used to calculate $\Delta^{17}\text{O}$ (Sharp et al., 2018; Whiteman et al., 2019). At the beginning of each analytical session, we measured a local water standard (NM2: $\delta^{18}\text{O} = -13.1\text{‰}$, $\delta^{17}\text{O} = -6.919\text{‰}$) that had been calibrated against the international water standards VSMOW2 ($\delta^{17}\text{O} = \delta^{18}\text{O} = 0.000\text{‰}$) and SLAP2 ($\delta^{18}\text{O} = -55.5\text{‰}$, $\delta^{17}\text{O} = -29.699\text{‰}$; Schoenemann et al., 2013; Sharp et al., 2016). The NM2 $\Delta^{17}\text{O}$ values associated with each measurement were then used to calculate a correction factor which we applied to the raw $\Delta^{17}\text{O}$ values of unknown samples to yield corrected values.

In addition to using $\Delta^{17}\text{O}$ values to understand reliance upon metabolic water, we used the combination of F_M values and $\delta^{18}\text{O}$ values of body water to calculate the $\delta^{18}\text{O}$ values of the combination of drinking and food water ($\delta^{18}\text{O}_{\text{D+PF}}$) that birds consumed as:

$$\delta^{18}\text{O}_{\text{DFW}} = \left[\delta^{18}\text{O}_{\text{BW}} - (F_M) \times (\delta^{18}\text{O}_{\text{Air}}) \right] / (1 - F_M) \quad (3)$$

Here, we assumed $\delta^{18}\text{O}_{\text{Air}}$ was 19.4‰ because of fractionation that occurs during absorption of inhaled atmospheric oxygen. This fractionation depends on the efficiency of oxygen absorption (EO_2 ; Epstein and Zeiri, 1988); although this efficiency was not measured in our experiment, previous research suggests that an EO_2 of 0.4 is reasonable for small passerines (Clemens, 1988; Arens and Cooper, 2005), which in humans produces

a fractionation of $\sim 4.4\text{‰}$ (Epstein and Zeiri, 1988). Although such data are lacking for our study species, applying the plausible range of fractionation values for absorbed oxygen (2–6‰) to equation 3 indicates that the resulting estimate of $\delta^{18}\text{O}$ of ingested water generally changes by $< 3\text{‰}$, which is smaller than much of the naturally occurring variation in $\delta^{18}\text{O}$ of potential water sources.

Metabolic Rates and Total Evaporative Water Loss

At the end of the 15-day experimental period at each temperature treatment (15°C or 30°C), we measured rates of oxygen consumption (VO_2) and TEWL for sparrows during 3–4 h using standard flow-through respirometry and hygrometry methods that we have previously applied to this species (Sabat et al., 2006a). Measurements were made at ambient temperatures (T_a) of $15.0 \pm 0.5^{\circ}\text{C}$ and $30.0 \pm 0.5^{\circ}\text{C}$ using an infrared O_2 - CO_2 analyzer equipped with a hygrometer (FMS, Sable Systems®). All trials were conducted in metallic metabolic chambers (2000 ml). Briefly, birds were placed in metabolic chambers kept at a constant temperature (15°C or 30°C) that received air free of water and CO_2 removed *via* Drierite and CO_2 absorbent at a flow of 750 ml/min ($\pm 1\%$). O_2 concentrations in the chamber were recorded during the active period between 06:00 and 18:00. Oxygen consumption was calculated according to the following equation (Lighton, 2018):

$$\text{VO}_2 = \text{FR} \times 60 \times (F_i \text{O}_2 - F_e \text{O}_2) / (1 - F_i \text{O}_2) \quad (4)$$

where FR is the flow rate in ml min^{-1} , and $F_i \text{O}_2$ and $F_e \text{O}_2$ are the fractional concentrations of inflow and outflow O_2 in the metabolic chamber, respectively. We calculated absolute humidity (kg/m^3) of air entering and leaving the chamber as $P/(T \times R_w)$, where P is water vapor pressure of the air in Pascal, T is the dewpoint temperature in Kelvin, and R_w is the gas constant for water vapor (461.5 J/kg K ; Lide, 2001). P was determined using the average value of the vapor pressure of the air entering the empty chamber (i.e., baseline period of 15 min) before and after each experiment with a dewpoint hygrometer located in the FMS. Total evaporative water loss was calculated as $\text{TEWL} = (V_e \times \rho_{\text{out}} - V_i \times \rho_{\text{in}})$, where TEWL is in mg/ml, ρ_{in} and ρ_{out} are the absolute humidity in kg/m^3 of the inlet air and the outlet air, respectively, V_i is the flow rate of the air entering the chamber as given by the mass flow controller (750 ml min^{-1}), and V_e is the flow of exiting air. V_e was calculated following (Williams and Tieleman, 2000) as:

$$V_e = V_i - [\text{VO}_2 \times (1 - \text{RQ})] + V_{\text{H}_2\text{O}} \quad (5)$$

V_{in} and VO_2 (ml min^{-1}) are known, and we assumed a respiratory quotient (RQ) of 0.71 (Sabat et al., 2006a). Output from the H_2O (kPa) analyzer, the oxygen analyzer (%), and the flow meter was digitalized using a Universal Interface II (Sable Systems, Nevada, United States) and recorded on a personal computer using EXPEDATA data acquisition software (Sable Systems, Nevada, United States). To estimate RMR, we averaged O_2 concentrations of the excurrent air stream

over a 20-min period after steady state was reached (Tieleman et al., 2002). We estimated the metabolic water production (WI_M) of sparrows using the equivalence of 0.567 ml H_2O per liter O_2 consumed (Schmidt-Nielsen, 1997). We calculated the $WI_M/TEWL$ ratio at different temperature treatments (15°C or 30°C). We also used equation (1) to calculate sparrow water balance, given that pre-formed water in food was negligible ($WPF=0$); after combining CWL and RWL into TEWL, and combining W_U and W_F into W_E as water losses via excreta, equation (1) was simplified to:

$$W_D + WI_M = TEWL + W_E \quad (6)$$

Birds were captured with permits from SAG, Chile (No. 10192/2019). All protocols were approved by the institutional Animal Care Committee of the University of Chile, following the recommendation of the ARRIVE guidelines (Kilkenny et al., 2010).

Statistical Analysis

We evaluated the effect of thermal acclimation on RMR, TEWL, and water intake using a generalized linear mixed model (GLMM) with body mass as a covariate, acclimation temperature (15°C and 30°C) as fixed factors, and individual identity as a random factor to control for repeated measures. Assumptions of normality and heteroscedasticity in residuals were examined with Q-Q plots and a plot of residuals against fitted values, respectively (Zuur et al., 2009). Dependent variables and covariates were natural log transformed for data normalization. Body mass and isotope values ($\Delta^{17}O$, $\delta^{18}O$, and $\delta^{17}O$) were compared between warm (30°C) and cold (15°C) treatments using non-parametric two-sample paired t-tests. The statistical analyses were performed in nlme package (Pinheiro et al., 2013) using the R platform (v4.0.3; R Development Core Team, 2013).

RESULTS

Physiological Data of Captive Sparrows

Sparrows acclimated at 15°C exhibited higher RMR (93.2 ± 15.2 ml O_2 h^{-1}) and lower daily water intake (0.19 ± 0.05 ml H_2O h^{-1}) in comparison with when they were acclimated at 30°C (RMR: 70.8 ± 12.2 ml O_2 h^{-1} and daily water intake: 0.26 ± 0.08 ml H_2O h^{-1}); however, there was no difference in TEWL between temperature treatments (Table 1). In addition, the $WI_M/TEWL$ ratio, converted to percentage, decreased significantly with increasing T_a (Figure 1), ranging from ~65% at 15°C to ~55% at 30°C. Using W_{IM} and W_D and the equation (6), metabolic water represented $22.8 \pm 4.2\%$ and $14.0 \pm 5.6\%$ of the total body water pool (i.e., $W_{IM}/(W_{IM} + W_D)$) at 15°C and 30°C respectively, while TEWL represented 80% of the total water loss at 15°C and 67% of the total water loss at 30°C.

Oxygen Isotopes

$\Delta^{17}O$ values were lower for captive sparrows acclimated to 15°C than 30°C (Wilcoxon Sign test = -7.5; $p=0.03$, Figure 2A).

$\Delta^{17}O$ -based estimates (via equation 2) of the proportion of metabolic water in the total body water pool in captive sparrows were 27.2 and 24.1% at 15°C and 30°C, respectively (Figure 2B). Wild *Cinclodes* $\Delta^{17}O$ values vary both within and among species (Figure 3), and $\Delta^{17}O$ -based estimates of the metabolic water contribution to the body water pool ranged from 19.6 to 31.0%. Mean ($\pm SD$) metabolic water contributions for *C. nigrofumosus* and *C. oustaleti* were $23.0 \pm 4.8\%$ and $27.7 \pm 4.0\%$, respectively. Intriguingly, two of the three highest measured $\Delta^{17}O$ values were sourced from *C. nigrofumosus*, suggesting a greater intake of pre-formed drinking/food water than for *C. oustaleti*. This inference is consistent with the general hypothesis of reduced dependence on WI_M for larger body-sized individuals across birds and mammals (Whiteman et al., 2019), because *C. nigrofumosus* (70–80 g) is more than twice the body mass of *C. oustaleti* (23–28 g; Sabat et al., 2006a). The mean ($\pm SD$) estimated $\delta^{18}O$ value of the combined pre-formed drinking/food water ingested by captive sparrows was $\sim -11 \pm 3\text{‰}$, which is within the range for tap water and groundwater in the Santiago Basin of central Chile (-15 to -11‰ ; Iriarte et al., 2004). In contrast, the pre-formed drinking/food water ingested by wild *Cinclodes* was estimated to have mean $\delta^{18}O$ ($\pm SD$) values near seawater (0‰; LeGrande and Schmidt, 2006): $0.2 \pm 8.3\text{‰}$ for *C. nigrofumosus* and $-1.4 \pm 0.9\text{‰}$ for *C. oustaleti*.

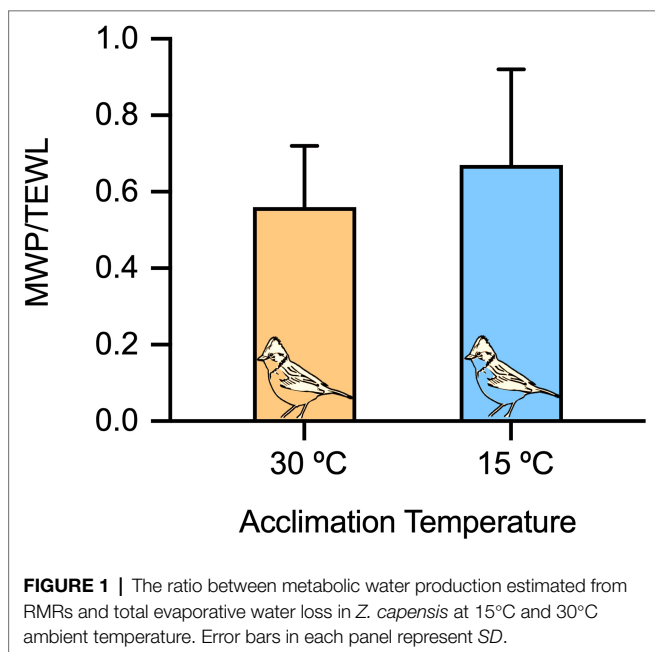
DISCUSSION

Metabolic water production (WI_M) alone is typically insufficient to meet the water requirements of most vertebrates (McNab, 2002), although some species adapted to arid environments or routinely experience long periods of time without food or drinking water can survive solely on metabolic water under certain conditions (Bartholomew and Cade, 1963; MacMillen and Hinds, 1983; Ostrowski et al., 2002). Several factors have been suggested to influence the importance of WI_M to the water budget of birds, such as the nature of oxidized substrates and environmental temperature. The current study aimed to experimentally assess the effects of changes in metabolic rate and water intake on $\Delta^{17}O$ values of captive house sparrows, kept in standard housing conditions at 30°C or 15°C for 15 days. As predicted, sparrows acclimated to cooler temperatures increased their RMR by ~31% while decreasing their drinking water intake by ~27% (Table 1). This elevation in metabolic rate suggests that an ambient temperature of 15°C is below their lower critical temperature (Maldonado et al., 2009). Accordingly, plasma $\Delta^{17}O$ values were lower in individuals housed at 15°C than at 30°C, reflecting the larger contribution of metabolic water relative to drinking/food water to the body water pool (Figure 2). These results support the predictions of a model of the relationship between the $WI_M/TEWL$ ratio and ambient temperature in granivorous birds (MacMillen, 1990). In this model, the ratio of $WI_M/TEWL$ increases when temperature declines below the T_{LC} , as a result of increased WI_M and dampened TEWL via water-recovery adaptations, such as desaturation of exhaled air into nasal surfaces (MacMillen, 1990). In a similar controlled experiment on mammals, $\Delta^{17}O$

TABLE 1 | Results of generalized linear mixed model testing the effect of 15 days of thermal acclimation to 15°C (cold) and 30°C (warm) on resting metabolic rate (RMR), total evaporative water loss (TEWL), and water intake in *Zonotrichia capensis*.

Predictors	Coeff. Estimate	95% CI	df	t	Value of p	R ² marginal/ conditional
RMR (ml O₂ h⁻¹)						0.58/0.58
Intercept	0.94	(-1.91–3.79)	9	0.75	0.47	
Mass	1.18	(0.22–2.13)	8	2.84	0.02	
Temperature (30°)	-2.25	(-0.40 to -0.10)	8	-3.85	0.005	
TEWL (mg H₂O h⁻¹)						0.10/0.25
Intercept	2.52	(-2.95–8.00)	9	1.04	0.32	
Mass	0.62	(-1.21–2.45)	8	0.78	0.45	
Temperature (30°)	-0.12	(-0.37–0.13)	8	-1.15	0.28	
Water Intake (ml H₂O)						0.25/0.66
Intercept	-2.45	(-8.96–4.06)	9	-0.85	0.41	
Mass	0.24	(-1.94–2.42)	8	0.26	0.80	
Temperature (30°)	0.34	(0.13–0.55)	8	3.67	0.006	

Numbers in bold indicate statistical significance ($p < 0.05$)



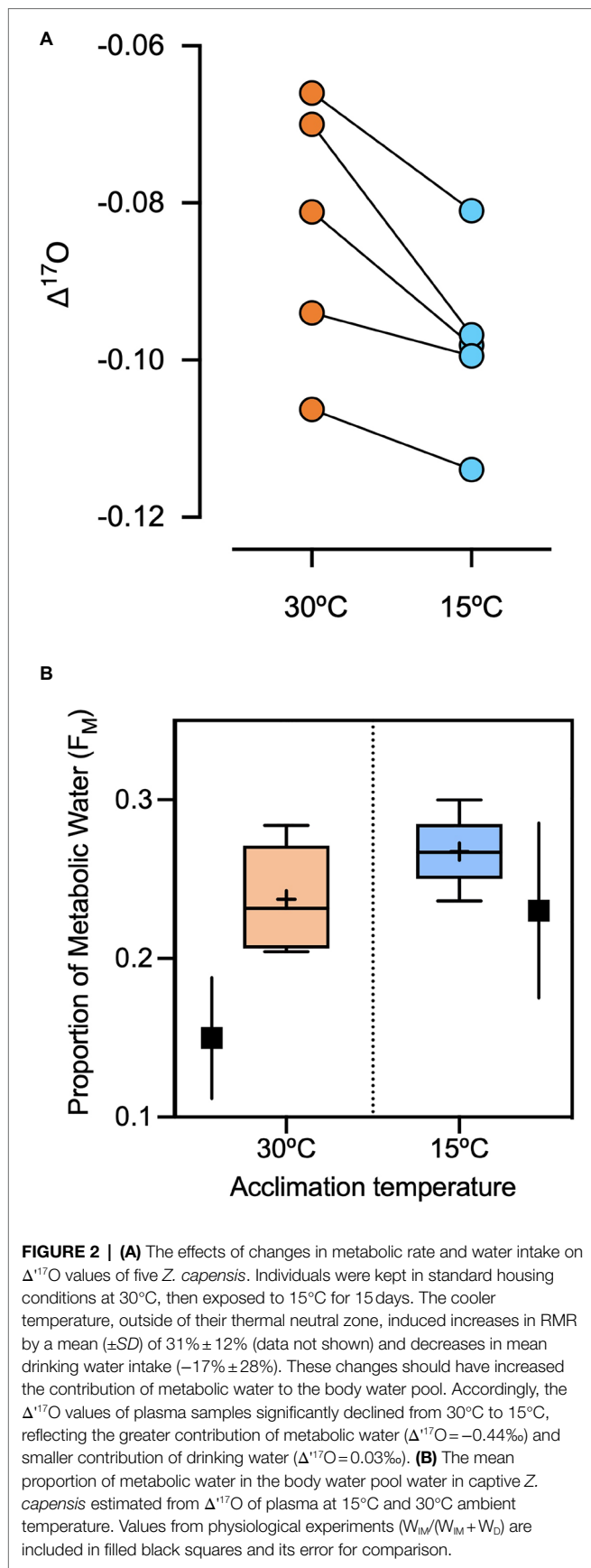
values of plasma from deer mice declined in response to elevated metabolic rate when animals were housed at 5°C rather than 25°C (Whiteman et al., 2019). The magnitude of the decline in $\Delta^{17}\text{O}$ (0.01–0.03 ‰) in deer mice was similar to that observed here in captive sparrows. Our results confirm that WI_M in small passerines increases with thermoregulatory demands and highlights the utility of this method for estimating water balance in laboratory conditions.

In a formative study, it was observed that the theoretical production of metabolic water can approach the rate of evaporative water loss in birds with body masses > 60 g, creating the opportunity for these larger birds to be “water-independent” and possibly rely solely on WI_M (Bartholomew and Cade, 1963). However, MacMillen (1990) suggested that smaller birds (e.g., < 20 g) can also attain favorable states of water balance ($\text{WI}_\text{M} > \text{TEWL}$) if they are below the lower critical temperature

(~25°C) when experiencing water deprivation. For the captive sparrows (~20 g) in our study, we calculated a mean $\text{WI}_\text{M}/\text{TEWL}$ of ~61% (Figure 1), which suggests that sparrows are not capable of relying solely on metabolic water production to maintain water balance, even in conditions that yield increases in metabolic rate (i.e., colder temperatures). Note that $\text{WI}_\text{M}/\text{TEWL}$ was 67 and 56% for cold- and warm-acclimated birds, respectively. Nevertheless, our estimates of TEWL come from an experimental setup that controlled the humidity inside the metabolic chambers near zero, which likely does not occur often in nature. In some birds, TEWL appears to vary as a function of absolute humidity ($\text{mg H}_2\text{O}/\text{m}^3$) across a range of environmental temperatures (Powers 1992, Gerson et al., 2014). By using data at comparable temperatures from the literature and climate data available from a local weather station,¹ we calculated that TEWL could be reduced on average by up to 20% at 30°C and 40% at 20°C, which would result in a $\text{WI}_\text{M}/\text{TEWL}$ of 76% at 30°C and up to 100% at 15°C.

The traditional approach to measuring WI_M is to assume a constant equivalence of water production based on oxygen consumption (Schmidt-Nielsen, 1997). Using this approach, we found that WI_M was 23% of the total water intake (i.e., $\text{WI}_\text{M} + \text{W}_\text{D}$) at 15°C but decreased to 14% at 30°C. These values are slightly lower to the percent contribution of metabolic water to the total body water pool that we estimated from $\Delta^{17}\text{O}$ data alone, which were 27.5% for cold-acclimated birds and 24.5% for warm-acclimated birds. The similarity in estimates of WI_M using the two approaches is notable because the $\Delta^{17}\text{O}$ estimates were based on collection of a single sample and did not rely on any measurements of water intake or loss, highlighting the potential accuracy of this new method (Whiteman et al., 2019). The discrepancy in estimates of WI_M , which is larger for warm-acclimated sparrows (14.0% vs. 24.5%), may be due to at least two non-exclusive alternatives. First, the calculation for the amount of water consumed per day was obtained under acclimatization conditions when birds were in larger cages that allowed for

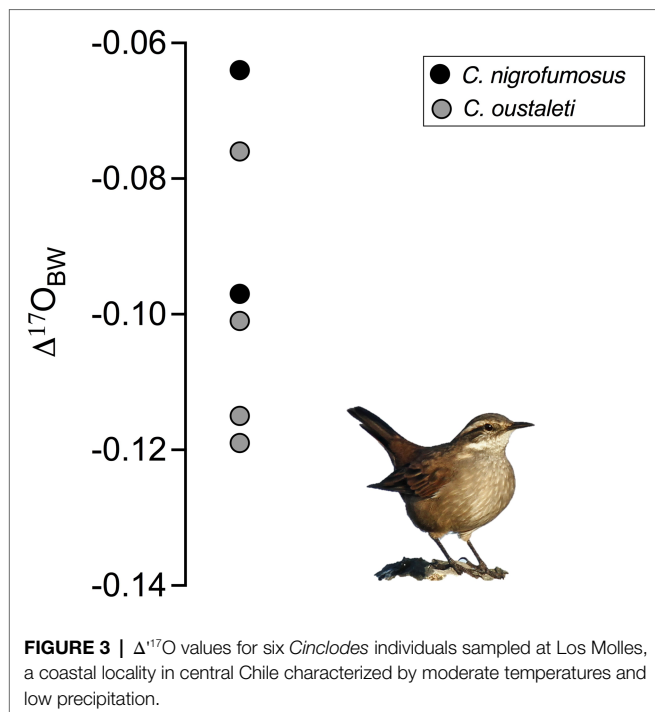
¹www.meteored.cl



movement and flight, conditions that yield higher rates of energy consumption than in the more confined conditions when RMR was measured. In addition, the traditional approach of using an equivalency between oxygen consumption and WI_{M} (i.e., 0.567 ml H_2O per liter O_2 consumed) does not distinguish between (1) H_2O that was produced by condensation reactions that occur during the oxidation of food that contains oxygen bound in macromolecules (e.g., protein or lipids) and (2) H_2O that was produced by complex IV of the electron transport chain, and which therefore only contains inhaled atmospheric oxygen (Morrison, 1953). In contrast, the $\Delta^{17}\text{O}$ approach only estimates the contribution of the latter mitochondrial source of H_2O . It should also be noted that body water exchanges oxygen atoms with dissolved CO_2 in the blood *via* the bicarbonate buffer system. This CO_2 is from metabolic decarboxylation (e.g., reactions that occur within the citric acid cycle) and contains oxygen bound in macromolecules (e.g., glucose) as well as phosphate groups (e.g., added by glucokinase). We expect that the influence of dissolved CO_2 on body water $\Delta^{17}\text{O}$ is small, because the isotopic fractionation associated with loss of exhaled CO_2 (Speakman and Racey, 1987; Haggarty et al., 1988) is mass-dependent, and because phosphate groups are likely in isotopic equilibrium with body water (Li et al., 2016). In general, more precise studies of water balance that considers the loss of water through urine and measurements of metabolic rate during longer periods in acclimatization conditions are necessary to establish the precision of the $\Delta^{17}\text{O}$ -based method.

The contribution of metabolic water to the body water pool is highly variable among birds, ranging from $< 10\%$ in some hummingbirds, $\sim 14\%$ in desert-adapted ostriches, and up to 80% in some passerines (MacMillen, 1990; Williams et al., 1993). For example, captive zebra finches (*Taeniopygia guttata*) with *ad libitum* access to drinking water produced only 1 ml metabolic H_2O per $\sim 1.5\text{--}1.8\text{ ml}$ of evaporative water lost at temperatures between 15 and 25°C, showing that without drinking water, the birds would have been in negative water balance. However, when birds were dehydrated for 30 days, their TEWL declined and $WI_{\text{M}}/\text{TEWL}$ increased to one (Cade et al., 1965). The influence of water availability on the relative importance of WI_{M} to total water pool in birds is in agreement with our previous work on small mammals. For example, captive mice (*Mus musculus*) that were provided drinking water *ad libitum* had smaller contributions of metabolic water to their body water than did wild desert-adapted small mammals (*Peromyscus leucopus*) of similar body mass (Whiteman et al., 2019). Overall, these results suggest that wild animals lacking *ad libitum* access to water responded by relying more on metabolic water than their captive counterparts.

Birds also have substantial flexibility in their sources of water intake. In wild zebra finches, WI_{M} calculated on the basis of field metabolic rate was lower during hot versus cool periods. As a consequence, WI_{M} fulfilled 20% of water requirements during hot days and 32% on cold days (Cooper et al., 2019). Because the total water turnover (ml/day) did



not vary substantially with environmental temperature, the change in the contribution of metabolic water to the total body water pool (i.e., F_M) is likely due to changes in metabolic rates. A question that remains unresolved is whether birds, especially species living in arid seasonal environments, modify their metabolic rate strictly for the purpose of WI_M . Lastly, another important variable that influences WI_M is the type of oxidative substrate used for aerobic metabolism. For instance, Zebra finches predictably lose body mass when fasting; however, when they are simultaneously water deprived, they lose substantially more body fat than lean (protein-rich) tissue (Rutkowska et al., 2016). The catabolism of body fat can potentially substantially increase the yield of metabolic water because fat is far more energy dense than other macromolecules oxidized for energy. In contrast, house sparrows (*Passer domesticus*) accelerate protein catabolism during acute dehydration (Gerson and Guglielmo, 2011), presumably to liberate pre-formed water molecules bound in proteinaceous (muscle) tissue (Giulivi and Ramsey, 2015).

We compared the $\Delta^{17}\text{O}$ -based estimates of the fractional contribution to body water from metabolic water (F_M) in captive sparrows with similar estimates for arid- and mesic-adapted wild birds based on allometric equations for water influx rate and field metabolic rate (Williams et al., 1993). We assumed that WI_M from oxidized substrates is 0.027 ml $\text{H}_2\text{O}/\text{KJ}$ based on oxidation of carbohydrates; note that this calculation yields the same result for WI_M as the equivalence method (0.567 ml H_2O per liter O_2) mentioned above (Morrison, 1953). We found that on average, the warm- and cold-acclimated sparrows in our study had F_M values that were ~29% and ~26% higher than the values expected for arid- and mesic-adapted birds of a similar

size. Estimates of F_M in wild *C. nigrofumosus* were ~23% or ~19% higher than expected values for similar-sized birds inhabiting an arid or mesic environment, respectively. Note that $\Delta^{17}\text{O}$ -based estimates of F_M for captive sparrows and wild *C. nigrofumosus* are within the 95% confidence interval of those reported for arid- and mesic-adapted birds of similar size. In contrast, $\Delta^{17}\text{O}$ -based estimates of F_M for *C. oustaleti* were significantly higher by ~48% or ~42% than predicted for birds from arid and mesic environments, respectively. We hypothesize that observed differences in F_M based on $\Delta^{17}\text{O}$ versus allometric proxies for *C. oustaleti* may be related to the high energetic and osmoregulatory costs of migrating between coastal (winter) and high elevation (summer) habitats, which may also be why this species has a relatively high BMR in comparison with other *Cinclodes* (Tapia-Monsalve et al., 2018). Overall, $\Delta^{17}\text{O}$ data revealed that the contribution of metabolic water to body water in captive sparrows and wild *Cinclodes* was similar to or within the same order of magnitude as for other free-ranging birds based on logarithmic allometric relationships. This finding emphasizes the validity of our method to the study of wild birds in natural ecosystems.

As expected, our regression approach based on oxygen isotope analysis shows that captive sparrows consumed drinking/food water that had a $\delta^{18}\text{O}$ value of -11‰, consistent with local tap water in Santiago, Chile (Figure 4). Likewise, isotope data show that wild *Cinclodes* consumed pre-formed drinking/food water with $\delta^{18}\text{O}$ values of ~0‰, which is consistent with the oxygen isotope composition of seawater (Figure 4). Intriguingly, one *C. nigrofumosus* individual ingested water with an anomalously positive estimated $\delta^{18}\text{O}$ value of 6.3‰ (Supplementary Table S1), notably higher than seawater. This enrichment could result from abiotic evaporation occurring in the environment prior to ingestion and/or physiologically mediated evaporative enrichment occurring within the organism. The first explanation suggests seawater ingested as pre-formed drinking/food water in the arid intertidal habitats where *C. nigrofumosus* forages in central and northern Chile may be ^{18}O -enriched. Alternatively, the relatively high $\delta^{18}\text{O}_{\text{DFW}}$ value observed for this species may result from isotopic fractionation that occurs during evaporation of body water, which largely depends on environmental temperature and humidity (Kohn, 1996). While *C. nigrofumosus* in this study was sampled in arid central Chile where evaporative ^{18}O -enrichment of body water could be a factor, this species inhabits humid coastal intertidal ecosystems and has ample access to drinking water in the form of seawater (Sabat et al., 2006a). In contrast to patterns for wild *Cinclodes*, we observed less within-treatment variation in estimates of $\delta^{18}\text{O}_{\text{DFW}}$ for captive sparrows (Supplementary Table S1), which is expected because (1) their drinking water was sourced from a municipal groundwater-derived aquifer with a relatively constant $\delta^{18}\text{O}$ value and (2) they were subjected to less variation in temperature and humidity in comparison with wild *Cinclodes* species (Sabat and del Rio, 2002; Sabat et al., 2006a). To better understand the ecological (e.g., habitat use and/or

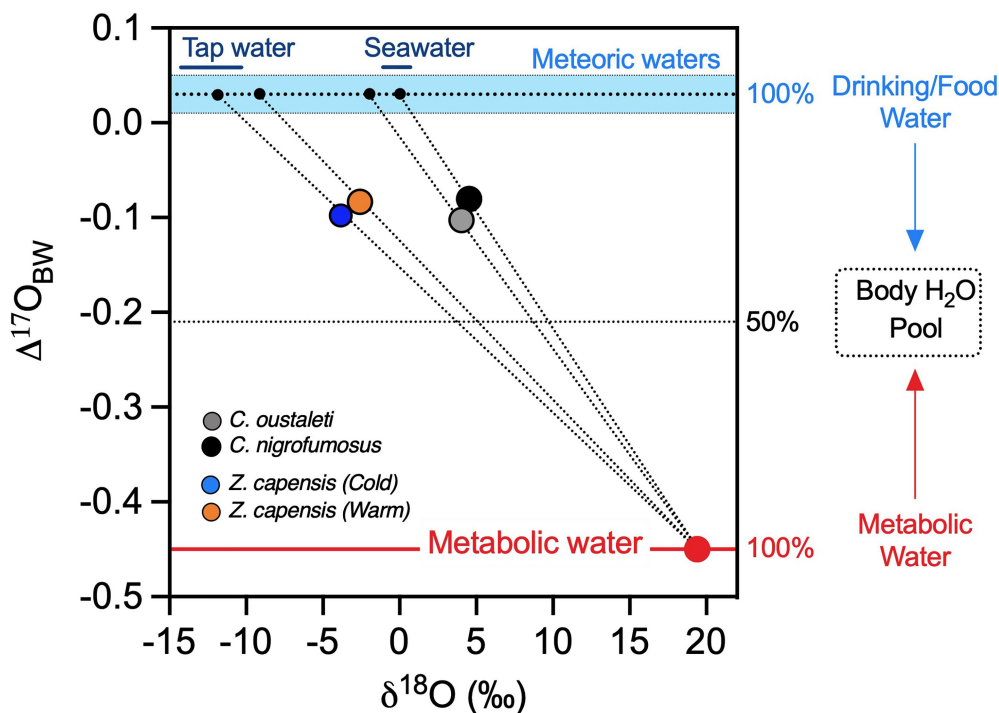


FIGURE 4 | Measured mean $\Delta^{17}\text{O}$ values (y-axis) and $\delta^{18}\text{O}$ values (x-axis) of body water used to estimate the $\delta^{18}\text{O}$ value of ingested drinking/food water of captive *Z. capensis* and wild *Cinclodes*. Captive *Z. capensis* was predicted to have ingested drinking/food water with a $\delta^{18}\text{O}$ value of -12‰ , which is consistent with tap water in Santiago, Chile. *Cinclodes* were predicted to have ingested drinking/food water with a $\delta^{18}\text{O}$ value of $\sim 0\text{‰}$, similar to seawater and consistent with their reliance on marine resources. Ranges of $\delta^{18}\text{O}$ values for tap water and seawater are shown by solid bars at the top of the range for meteoric waters (sky-blue dashed area). The dotted lines represent the solutions for equation 2 for each group of birds.

diet composition) and environmental (e.g., temperature and/or humidity) factors that influence water budgets in birds will require more experiments that assess the effect of physiologically mediated water conservation strategies on the oxygen isotope composition of body water, and additional sampling of birds from a range of environments that span temperature and humidity gradients.

Finally, apparent differences in $\delta^{18}\text{O}$ of pre-formed drinking/food water between *Cinclodes* species may be related to inter-specific variation in the ability to cope with saline-rich prey and drinking water sources (Sabat et al., 2006b; Tapia-Monsalve et al., 2018). For example, the high salinity tolerance of *C. nigrofumosus* (Sabat et al., 2006b), especially at the northern margin of its distribution, may allow this species to rely less on metabolic water because of the ability to obtain and process an abundant source of drinking/food water sourced from the ocean. The ecological conditions that either favor or limit the ability of birds to depend on metabolic water remain largely unknown, while the combined effect of temperature and humidity on a bird's water budget is poorly understood, especially in free-ranging individuals. We predict that a species dependence on metabolic water vs. pre-formed drinking/food water along environmental gradients will depend on the interaction between the ability of different populations/species to retain water and dissipate heat, coupled with the availability of pre-formed water as well as environmental temperature and humidity. For example,

the study of bird species and/or populations that differ in how they evaporatively cool their bodies *via* panting, hyperthermia, or cutaneous evaporation that ultimately influences water loss rates (Gerson et al., 2014) represents a unique opportunity to determine which environmental and physiological variables modulate an animal's use of metabolic versus pre-formed water to maintain water balance.

Overall, our study revealed that the contribution of metabolic water to the total body water pool increased with metabolic rate, consistent with a recent report in small mammals that used a similar $\Delta^{17}\text{O}$ -based approach (Whiteman et al., 2019). Although these increases may not seem very significant in magnitude, they could account for natural changes in the energy expenditure of animals in the field. More precise studies of water balance that considers all potential sources of water losses during longer periods in acclimatization are necessary to establish the precision of the $\Delta^{17}\text{O}$ -based method used in this study. $\Delta^{17}\text{O}$ -based estimates of F_M in captive sparrows and wild *C. nigrofumosus* were similar to those for free-ranging birds based on independent allometric relationships, while estimates for *C. oustaleti* based on oxygen isotopes were higher than expected, but in the same order of magnitude as predictions based on logarithmic allometric relationships, emphasizing the validity of our method to better understand water balance in wild birds.

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 all protocols were approved by the institutional Animal Care Committee of the University of Chile (CICUA), and National Research and Development Agency (ANID).

AUTHOR CONTRIBUTIONS

PS, SN, and JW designed the research. SP, PS, ZS, and JW performed the research. SP, AG, KM, and PS analyzed the data. PS, SN, RN, JS-H, KM, and JW wrote the paper. 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/fphys.2021.710026/full#supplementary-material>

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Integrative Physiological Responses to Acute Dehydration in the Rufous-Collared Sparrow: Metabolic, Enzymatic, and Oxidative Traits

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Predictions indicate that birds worldwide will be affected by global warming and extreme climatic events which is especially relevant for passerines because the diurnal habits, small body size, and high mass-adjusted metabolic rates of this group make it particularly susceptible to increases in temperature and aridity. Some bird species respond to conditions that stress osmoregulation by increasing their rates of energy expenditure, nevertheless, the effect of dehydration on metabolic rates in birds has produced contrasting results. It also remains unknown whether hydration state may cause shifts in tissue-specific metabolic rates or modify tissue oxidative status. We used the rufous-collared sparrow (*Zonotrichia capensis*), to experimentally test the effect of dehydration on metabolic enzymes in erythrocytes, tissue oxidative status, basal metabolic rate (BMR), and total evaporative water loss. We found a significant increase in mass-adjusted BMR in water restricted (WR) birds compared to control birds (CT). Activity of cytochrome-c-oxidase (COX) in red blood cells (RBCs) was also significantly higher in the WR group relative to the CT group and this activity was positively correlated with mass-adjusted BMR. We found a moderate effect of water restriction on membrane damage of skeletal muscle. In a second set of individuals subjected to the same experimental conditions, lean mass and total water were tightly correlated and decreased by 10 and 12%, respectively, in birds in the WR group relative to the CT group. Decreases in total water and lean mass leads to an increase in mass-adjusted BMR in WR *Z. capensis*, suggesting that birds may simultaneously increase protein catabolism and production of metabolic water through oxidation. The significant positive relationship between BMR and COX in RBCs is a finding that requires additional research to determine whether erythrocyte metabolism is affected by dehydration *per se* and or it more generally reflects rates of energy expenditure in birds.

Keywords: basal metabolic rate (BMR), body composition, metabolic enzymes, oxidative stress, birds, dehydration

INTRODUCTION

Increased frequency in droughts and heat waves are predicted to have a strong influence on plant and animal phenology, behavior, and physiology (Walther et al., 2002; Albright et al., 2017). Although predictions indicate all birds worldwide will be affected by global warming and climatic extreme events, species that live in subtropical and temperate deserts will be disproportionately impacted by climate change because the magnitude and perhaps rate of change in these regions will likely be larger and faster than in other biomes (McKechnie and Wolf, 2010; Albright et al., 2017; Iknayan and Beissinger, 2018). This is especially relevant for passerines because the general behavioral (e.g., diurnal), evolutionary (e.g., small body size), and physiological (e.g., high mass-specific metabolic rates, dissipate heat via panting) traits of this group make it particularly susceptible to increases in temperature and aridity (Yom-Tov, 2001; Gardner et al., 2011; Albright et al., 2017). To better assess how rapid environmental changes threatens species' persistence, phenomenological data should be combined with laboratory experiments to identify the mechanisms that control how animals respond to environmental conditions (Khaliq et al., 2014).

Maintaining osmotic balance is a challenge when birds are feeding on salty food or living in arid habitats that contain scarce freshwater (Sabat and Martinez del Rio, 2002; Sabat et al., 2009; Smit and McKechnie, 2015). Avian water balance is a function of the interplay between water availability in the environment, the physiological and behavioral mechanisms birds use to reduce water losses, and the production of metabolic water that is directly linked to metabolic rate (Gerson and Guglielmo, 2011; Albright et al., 2017; Sabat et al., 2021). Some bird species respond to environmental conditions that stress osmoregulation by increasing their rates of energy expenditure, including basal metabolic rate (BMR). This response has been associated with an increase in hyperosmotic urine excretion, which in turn comprises both the energy cost of osmoregulation *per se* (McWhorter et al., 2004) and the long-term effect of increasing the mass and metabolic intensity of tissues such as the kidneys and heart (Daan et al., 1990; Gutiérrez et al., 2011; Peña-Villalobos et al., 2013; Sabat et al., 2017). Nevertheless, the effect of dehydration on metabolic rates in birds has produced contrasting results (Cade et al., 1965; Gerson and Guglielmo, 2011).

Another potential cost of dehydration could be the oxidative stress that results from maintaining high metabolic rates. Mitochondria are the principal source of cellular reactive oxygen species (ROS) associated with the production of superoxide anions as byproducts of the electron transport chain and other redox reactions (Srinivasan and Avadhani, 2012). Several studies have reported that increases in metabolic rate results in higher production of ROS, which consequently stimulates energetically intensive antioxidant defense (Commoner et al., 1954; Cohen et al., 2008; Tumminello and Fuller-Espie, 2013). Moreover, some studies have shown that osmotic stress affects several physiological traits in birds, including shifts in tissue-specific metabolic rates and oxidative status (Tieleman et al., 2003; Tapia-Monsalve et al., 2018; Sabat et al., 2019). In support of this

mechanism, a recent study revealed that baseline corticosterone levels were higher in water-deprived house sparrows (Brischoux et al., 2020), and glucocorticoid has been shown to disturb cellular oxidative homeostasis (Costantini et al., 2011; Queisser et al., 2011). Furthermore, recent evidence indicates that dehydration induced by high environmental temperatures influences cellular metabolism in mammals, ultimately yielding an elevated antioxidant response and/or oxidative damage (Jacobs et al., 2020). It remains unknown whether hydration state and changes in osmotic conditions may cause oxidative damage in birds. We predicted that a potential additional cost of dehydration is the increased production of ROS, which leads to cumulative damage of biomolecules, Costantini et al. (2010).

Cells can shrink in response to increases in blood plasma osmolality by hydric stress, and erythrocytes must maintain their volume by means of energy intensive ion regulation (Hoffmann et al., 2009; Gerson and Guglielmo, 2011). Birds have functional mitochondria in their red blood cells (RBCs) containing specific enzymes that provide immediate energy for ion-transporting proteins (Moritz et al., 1997; Stier et al., 2013). Recent studies have documented the existence of significant inter-individual variation in the energy expenditure rates of erythrocyte mitochondria in birds. Specifically, variation in mitochondrial metabolism is associated with the activity of the metabolic enzymes Citrate Synthase (CS) and Cytochrome C Oxidase (COX) in skeletal muscle (Stier et al., 2015, 2017). These two enzymes are responsible for energy (ATP) production such that the activity of these enzymes is correlated with whole-body energy consumption rates in birds (Peña-Villalobos et al., 2014; Gutiérrez et al., 2019). Thus, it is possible that the metabolism of the mitochondria, through the expression of enzymes involved in the expenditure of energy at the tissue and peripheral level, is correlated with physiological modifications that occur in birds subjected to water stress.

Here we integrated blood-based measurement of energy metabolism (e.g., CS and COX) with oxidative status (e.g., lipid peroxidation and glutathione balance) and BMR to examine the osmoregulatory adjustments to dehydration in captive rufous-collared sparrows (*Zonotrichia capensis*), a common passerine bird inhabiting southern South America. Specifically, we hypothesize that birds exposed to water restriction for 2 weeks will show higher plasma and urine osmolality, activity of the mitochondrial enzymes, and metabolic rates than hydrated (control) birds. We also predict that water restricted (WR) birds should exhibit a higher ratio of oxidized (GSSG) to reduced (GSH) glutathione; higher lipid peroxidation (MDA concentration) as a measurement of oxidative damage, higher of hydrogen peroxides (H_2O_2) concentrations, and enhanced total antioxidant capacity (TAC).

MATERIALS AND METHODS

Laboratory Acclimation

A total of 18 *Z. capensis* (Passeriformes: Emberizidae) individuals were captured in November 2018. Captive birds were held for 2 weeks at a constant temperature of $25 \pm 2^\circ\text{C}$ within

their thermoneutral zone (TNZ; Sabat et al., 2019) and housed individually in plastic-mesh cages (35 cm × 35 cm × 35 cm) under a 12L:12D photoperiod. After capture, each bird was assigned to one of two treatments: a control (CT) group was maintained with water and seeds *ad libitum* and a WR group with seeds *ad libitum* that during the first week were allowed to drink fresh water every 24 h, and the second week every 48 h. Individuals were weighed with a scale (± 0.001 g) at the beginning and end of each week of treatment. Birds were captured with permits issued by Servicio Agrícola Ganadero (No. 10192/2019). All protocols were approved by the institutional Animal Care Committee of the University of Chile where experiments were performed.

Basal Metabolic Rate and Total Evaporative Water Loss

Immediately after each treatment, we measured BMR ($\text{mL O}_2 \text{ h}^{-1}$) and total evaporative water loss (TEWL) in post-absorptive (fasted for 4-h) resting birds during the night between 21:00 and 07:00 h using standard flow-through respirometry methods. Respirometry measurements were performed on three birds per night and staggered such that the beginning of the water restriction experiments started sequentially. Oxygen consumption was measured using three computerized open-flow respirometry systems (two FMS, one FOXBOX, Sable Systems, Henderson, NV, United States) calibrated with a known mix of oxygen (20%) and nitrogen (80%) that were certified by chromatography (INDURA, Chile) and then placed in a controlled temperature cabinet (Sable Systems, Henderson, Nevada) at a constant ambient temperature of $30 \pm 0.5^\circ\text{C}$ that is within the TNZ for this species (Sabat et al., 2019). The metabolic chamber received dried air at 500 mL min^{-1} through Bev-A-Line tubing (Thermoplastic Processes Inc) from a mass flow controller. The excurrent air passed through a hygrometer that recorded vapor pressure (kPa) and then through columns of Baralime to absorb CO_2 before passing through the O_2 -analyzer. Because CO_2 was scrubbed before entering the O_2 analyzer, oxygen consumption was calculated as (Withers, 1977): $\dot{V}\cdot\text{O}_2 = [\text{FR} \times 60 \times (\text{Fi O}_2 - \text{Fe O}_2)] / (1 - \text{Fi O}_2)$, where FR is the flow rate in mL/min , Fi and Fe are the fractional concentrations of O_2 entering and leaving the metabolic chamber, respectively. TEWL was calculated as $\text{TEWL} = [(\text{Ve } \rho_{\text{out}} - \text{Vi } \rho_{\text{in}})]$ where TEWL is in mg/mL . ρ_{in} and ρ_{out} are the absolute humidity in kg/m^3 of the inlet air and the outlet air, respectively, Ve is the flow rate of the air entering the chamber as given by the mass flow controller equipped with the FMS and FOXBOX (500 mL min^{-1}) and Ve is the flow of exiting air. Ve was calculated following (Tieleman et al., 2003) as $\text{Ve} = \text{Vi} - [\dot{V}\cdot\text{O}_2 (1 - \text{RQ})] + \text{VH}_2\text{O Vi}$, and $\dot{V}\cdot\text{O}_2$ (mL min^{-1}) are known. We used a respiratory quotient (RQ) of 0.71, which assumes that fasting sparrows rely mainly on stored lipids for metabolism (King and Farnier, 1961; Walsberg and Wolf, 1995). Absolute humidity was calculated as $\rho = P/(T \times R_w)$, where P is water vapor pressure of the air in Pascal, T is the temperature of the dew-point hygrometer in Kelvin and R_w is the gas constant for water vapor [$461.5 \text{ J/kg} \times \text{Kelvin}$, (Lide, 2001)]. The ρ_{in} was determined

using the average value of the vapor pressure of the air entering the empty chamber during the 15-min baseline period before and after each experiment. Body mass was measured before the metabolic measurements using an electronic balance (± 0.1 g) and cloacal body temperature (T_b) was recorded with a thin Cole-Palmer copper-constantan thermocouple attached to a Digisense thermometer (Model 92800-15) within a minute after the birds were removed from metabolic chamber. We found that all animals were euthermic after the metabolic trials ($T_b > 40^\circ\text{C}$). Data readings were performed through EXPEDATA software (Sable Systems, International, NV, United States). To estimate basal metabolic rate and TEWL, we averaged O_2 concentrations and water vapor pressures of the excurrent air stream over a 20 min period after steady state was reached, which typically occurs after 3 h in *Z. capensis* (Maldonado et al., 2009). We estimated the metabolic water production (MWP) of sparrows using the equivalence of $0.567 \text{ mL H}_2\text{O}$ per liter O_2 consumed (Schmidt-Nielsen, 1997). We calculated the ratio between MWP and water losses (MWP/TEWL) for the 20 min period in which the steady state was reached.

Blood and Urine Osmolality

Blood ($50\text{--}100 \mu\text{L}$) was collected in the morning (09:00–11:00 h) prior to physiological measurements from the humeral vein using hematocrit tubes with anticoagulant (heparine or EDTA). Blood samples were centrifuged at $10,000 g$ for 5 min to separate plasma from RBCs, and both fractions were frozen at -80°C until analysis. Ureteral urine was obtained by inserting a small closed-ended cannula into the bird's cloaca, which was centrifuged, and the supernatant was collected for osmometry analysis (Wescor 5130B). After metabolic measurements, birds were sacrificed by CO_2 exposure, weighed, and the organs were removed and frozen at -80°C .

Metabolic Enzymes

Blood pellets consisting of the cellular fraction comprised mostly of RBCs was thawed and transferred to a Falcon tube, weighed in an analytic balance (± 0.0001), and homogenized on ice (1:4 w/v) in phosphate buffer 0.1 M supplemented with 2 mM EDTA (pH 7.3). To obtain similar masses of RBC isolates across samples, the weight of a given RBC pellet was corrected by the value of the mean hematocrit of all samples (i.e., $w \times [\text{sample hematocrit}/\text{mean hematocrit}]$). The samples were then sonicated using a 130 Watt Ultrasonic Processor (VCX 130) on ice 14 times in 20-s cycles and 10-s interval between cycles. Homogenates were then centrifuged at $14,000 \text{ rpm}$ for 15 min at 4°C to obtain a post mitochondrial fraction. The supernatant was transferred into a new tube to avoid transferring the upper lipid layer present in the homogenate. Protein concentrations were determined using the method by Bradford (1976) with bovine serum albumin as the standard. We measured the activity of two mitochondrial enzymes: COX (E.C. 1.9.3.1) and CS (E.C. 4.1.3.7). COX activity was quantified using a microplate spectrophotometric method in which enzyme activity was measured in a reaction mixture containing 10 mM Tris-HCl (pH 7), 120 mM KCl, 250 mM sucrose, and cytochrome c reduced with dithiothreitol in a final volume of 0.2 mL. The decrease in extinction at 550 nm

was monitored in a Thermo Scientific Multiskan GO UV/VIS spectrophotometer at 25°C. COX activity was calculated using an extinction coefficient of $21.84 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550 nm. To quantify CS, the enzyme assay medium contained 10 mM Tris-HCl (pH 8.0), 10 mM 5,5'-dithiobis- (2 nitrobenzoic acid), 30 mM acetyl Coenzyme A (acetyl CoA) and 10 mM oxaloacetic acid (OAA) in a final volume of 0.2 mL; these reagents were omitted in controls. CS catalyzes the reaction between acetyl CoA and OAA to form citric acid. The increase in extinction at 412 nm was measured in a Thermo Scientific Multiskan GO at 25°C. CS activity was calculated using an extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 412 nm (Peña-Villalobos et al., 2014).

Oxidative Stress

Tissues were thawed and processed following the specific procedures for each analytic protocol (see below). Oxidative stress was evaluated by measuring the concentration of reduced (GSH) and oxidized (GSSG) glutathione in pectoralis and liver. Lipid peroxidation (TBARS) was measured in plasma, liver, heart, pectoral, and RBCs. Hydrogen peroxides (H_2O_2) were measured in plasma, liver, heart and pectoralis. Finally, we measured TAC in plasma, heart and pectoralis.

Concentration of reduced (GSH) and oxidized (GSSG) glutathione was measured in the acid supernatants, which were obtained by treatment of the post-mitochondrial fraction (200 μL) with 200 μL of cold 0.6% (w/v) sulfosalicylic acid, and centrifuged ($10,000 \times g$, 5 min, 4°C) after keeping the samples for 5 min on ice. Determination of GSH and GSSG concentrations followed the colorimetric method by Rahman et al. (2006), adapted to microplate format. Lipid peroxidation was measured in plasma, liver, heart, pectoral, and RBCs (EF) with a commercial kit Oxiselect, STA-330 Cell Biolabs; (Gutiérrez et al., 2019). The assay evaluates the joint between adduct. Absorbance was measured in a Thermo Scientific Multiskan GO UV/VIS spectrophotometer at 25°C. We measured hydrogen peroxides (H_2O_2) in plasma as a measure of the pro-oxidant status (Costantini, 2014) using a commercial kit (MAK311, Sigma Aldrich). The assay evaluated colorimetrically the oxidation of Fe^{+2} to Fe^{+3} by peroxides present in the sample at 585 nm. We also measured antioxidant capacity with the adjustment for microtiter plate (Ribeiro et al., 2011). In brief, the assay measures the reduction of Cu (II)-neocuproine complex to Cu(I)-neocuproine complex by antioxidants present in the sample, the assay is measured spectrophotometrically at 450 nm, and the sample value is compared with a known Trolox standard curve.

Quantitative Magnetic Resonance

To estimate the effect of dehydration on body composition, we repeated the experiment of water restriction on a second set of *Z. capensis* ($n = 11$) captured from the same locality and subject to the same experimental conditions. For logistical reasons, we could not analyze the body composition of birds in the initial experiment. At the end of this second acclimation experiment, on the night after the last dehydration period (i.e., Day 14), birds were transported to the Universidad Austral de Chile in Valdivia (~850 Km from Santiago, 10 h of travel) and inserted into a EchoMRI 500 (Houston, TX, United States)

quantitative magnetic resonance (QMR) instrument to directly measure grams of lean mass, fat mass, and total, and free body water. This instrument has been validated in wild animals (Riley et al., 2016; Kraft et al., 2019; Eastick et al., 2020) and has been specifically used to measure changes in lean and fat mass during migration in birds (Guglielmo et al., 2011; Kelsey and Bairlein, 2019). For each measurement, the animal was placed in an acrylic cylinder (5 cm diameter, 60 cm long) and immobilized by a Velcro-secured plunger placed inside the cylinder. The cylinder was then placed into the magnetic resonance module, which was programmed for three scans per animal achieved in ~1.5 min. If the coefficient of variation of these scans exceeded 6%, usually due to movement of the animal within the probe, the measurement was discarded and repeated. We calibrated the QMR daily before every batch of measurements according to manufacturer's recommendation by using a sample of canola oil of known mass. We calculated a hydration index for each individual birds as: total water – free water/lean mass.

Statistical Analysis

We used Repeated Measures Analysis of Variance (RM-ANOVA) and *a posteriori* Fisher test to assess differences in body mass between the experimental treatments. The same procedure was used for the second experimental group of birds used to estimate body composition. We evaluated the effect of water availability on BMR, TEWL, organ masses, biomarkers of oxidative stress, and fluid concentrations using a general linear model (GLM) with final body mass as a covariate and hydration state as fixed factors. We initially included a treatment by body mass interaction, but this interaction was not significant and removed from the linear model. We then tested the effect of the covariates body mass and treatment again, and when the covariate was non-significant, we removed this term from the model. To estimate the extent to which metabolic enzyme activity is associated with body mass and metabolic rate, we ran a multiple linear regression using whole BMR as the response variable and enzyme activities and body mass as predictors. Finally, we calculated and plotted the residuals of the linear regression between body mass and enzyme activities. All statistical analyses were performed using STATISTICA® statistical package for Windows. For simplicity, here we only present results from analyses using the covariate when the GLM revealed a significant effect of body mass. Detailed results of the effects of body mass and the interaction between body mass and treatment are reported in **Supplementary Table 1**.

RESULTS

Body and Organ Mass

We found a significant effect of experimental time on body mass [RM ANOVA, $F_{(3,48)} = 47.48$; $p < 0.01$], with a steeper decline in body mass over time in the WR group (**Figure 1**) such that by day 7 the two treatments significantly differed from one another [$F_{(1,16)} = 25.82$; $p < 0.01$, **Figure 1**]. We also found an interaction between time and treatment on body mass [$F_{(3,48)} = 12.05$; $p < 0.01$], with the WR group being the most affected and losing up to 17.7% of initial mass on average, while the CT group only

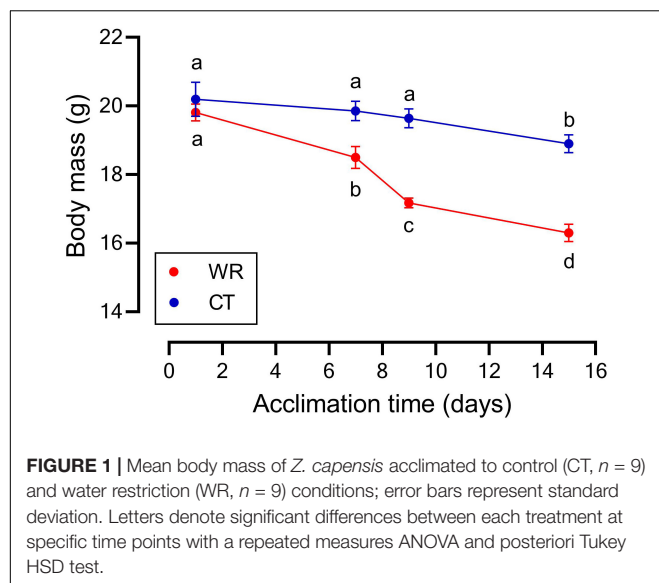


TABLE 1 | Organ masses (means and LSM $\pm$ standard error) of *Z. capensis* acclimated to hydrated (control) and water restriction conditions (CT = control; $n = 9$ and WR = water restricted; $n = 9$).

	CT		WR	
	Mean	LSM	Mean	LSM
Heart (g)	0.23 $\pm$ 0.011	0.19 $\pm$ 0.01*	0.20 $\pm$ 0.01	0.23 $\pm$ 0.01
Liver (g)	0.57 $\pm$ 0.041	0.49 $\pm$ 0.04	0.41 $\pm$ 0.02	0.49 $\pm$ 0.04
Intestine (g)	0.46 $\pm$ 0.03 (71.28%)	0.39 $\pm$ 0.02	0.34 $\pm$ 0.02 (72.56%)	0.40 $\pm$ 0.03
Kidney (g)	0.20 $\pm$ 0.01	0.18 $\pm$ 0.01*	0.21 $\pm$ 0.01	0.22 $\pm$ 0.01
Pectoral (g)	0.85 $\pm$ 0.12 (76.77%)	0.66 $\pm$ 0.11	0.63 $\pm$ 0.03 (75.50%)	0.82 $\pm$ 0.11

Asterisk denotes significant differences in LSM between experimental groups after an ANCOVA (see **Supplementary Table 1B** for details). The mean percentage of water for intestine and pectoral muscle are provided in parentheses.

decreased by 6.4% (**Figure 1**). The masses of internal organs were all correlated with final body mass (see **Supplementary Table 1B**) and the ANCOVAs showed that when body mass was accounted for the masses of heart [$F_{(1,15)} = 5.67$; $p = 0.030$] and kidneys [$F_{(1,15)} = 9.621$; $p = 0.008$] were influenced by water restriction, with the WR group having larger organs than the CT group (**Table 1** and **Supplementary Table 1B**).

Quantitative Magnetic Resonance Measurements

At the end of the second acclimation experiment, birds in the WR group had $\sim 12\%$ lower body mass [$F_{(1,9)} = 10.79$; $p < 0.001$], 10% lower lean mass [$F_{(1,9)} = 11.72$; $p = 0.008$], and 11% lower total water content [$F_{(1,9)} = 13.97$; $p = 0.005$] in comparison to the CT group (**Table 2**). No significant differences were found in fat mass [$F_{(1,9)} = 0.07$; $p = 0.79$] and free water content among treatments [$F_{(1,9)} = 2.04$; $p = 0.18$]. The calculated hydration index values were 2% higher in the CT versus WR group [$F_{(1,9)} = 18.59$; $p = 0.002$]. Finally, we found a strong and positive

relationship between total body water and lean mass when all individuals were pooled ($r = 0.98$; $p < 0.001$).

Physiological Responses

Concentration of body fluids were not affected by body mass (**Supplementary Table 1C**). Blood plasma osmolality did not significantly differ between the experimental treatments [$F_{(1,14)} = 1.70$; $p = 0.21$ **Figure 2**], however, at the end of the 2-week acclimation period we observed a significant increase of $\sim 57.0\%$ [$F_{(1,14)} = 123.2$; $p = 0.001$] in urine osmolality in the dehydrated relative to the hydrated treatment (**Figure 2**).

Sparrows in WR group had $\sim 12\%$ lower whole-organism TEWL in comparison to birds in the CT group [$F_{(1,16)} = 5.42$, $p = 0.033$], however, there was no difference in whole-organism BMR between experimental treatments [$F_{(1,16)} = 1.85$, $p = 0.192$; **Table 2**]. Both BMR and TEWL were significantly correlated with body mass ($r^2 = 0.39$; $p = 0.005$, $r^2 = 0.282$; $p = 0.023$ for BMR and TEWL, respectively, **Figure 3**) although, the GLM revealed that only BMR was significantly correlated with body mass (**Supplementary Table 1A**). GLM analyses showed that the mass-adjusted BMR (least square means, LSM) of the WR group was $\sim 12\%$ higher on average than the BMR of the CT group [$F_{(1,15)} = 4.84$; $p = 0.044$, **Table 3**].

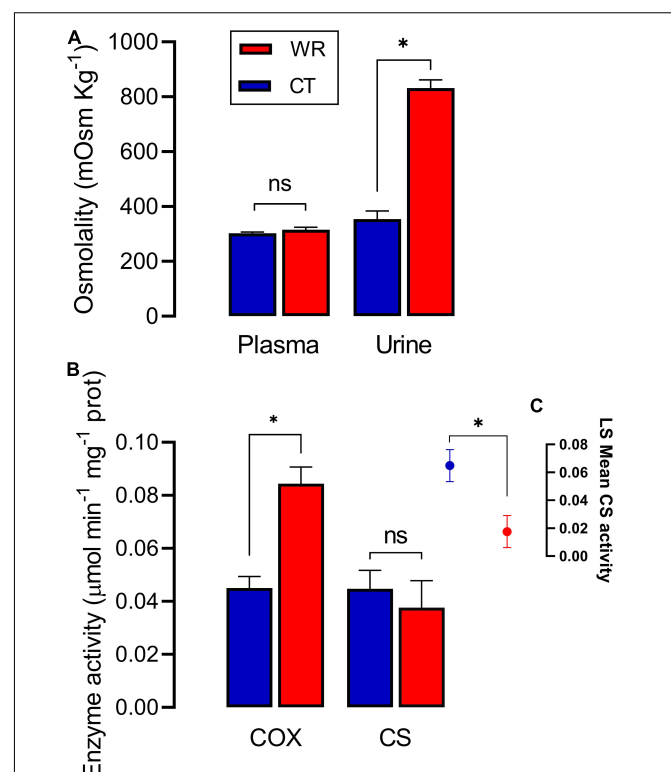


TABLE 2 | Mean ($\pm$ SE) of body composition in *Z. capensis* acclimated to water restriction (WR) and control (CT) conditions for 15 days.

	Body mass (g)	Fat (g)	Lean (g)	Free H ₂ O (g)	Total H ₂ O (g)	Hydration index
CT	20.53 $\pm$ 0.49*	0.12 $\pm$ 0.08	16.24 $\pm$ 0.31*	0.43 $\pm$ 0.032	14.35 $\pm$ 0.28 *	0.859 $\pm$ 0.003
WR	18.12 $\pm$ 0.54	0.15 $\pm$ 0.09	14.66 $\pm$ 0.34	0.36 $\pm$ 0.036	12.79 $\pm$ 0.31	0.842 $\pm$ 0.003

Asterisks denote significant differences between mean values of experimental groups after ANOVA.

After correcting for body mass, the activity of CS in erythrocytes was significantly higher in the CT than in the WR treatment [$F_{(1,15)} = 5.49$, $p = 0.030$; **Figure 2**], and the average activity of the metabolic enzyme cytochrome-c-oxidase was significantly higher in the WR than in the CT treatment [$F_{(1,16)} = 26.08$, $p < 0.001$, **Figures 2, 3** and **Supplementary Table 1A**]. The GLM analysis on pooled data revealed that whole animal BMR was affected by body mass [$F_{(1,16)} = 37.70$, $p < 0.0001$], COX activity [$F_{(1,16)} = 17.93$, $p = 0.001$], but not by CS activity [$F_{(1,16)} = 0.10$, $p = 0.75$]. We also observed a significant and positive correlation between the residuals of the relationships between body mass versus BMR or COX activity ($y = 279.0x + 0.00$; $r^2 = 0.560$, $p < 0.001$; **Figure 4**).

Oxidative Status

Concentrations of markers of oxidative status were significantly different among tissues (**Figures 5, 6**), but there was no effect of body mass on these parameters except for glutathione concentrations in pectoral muscle (see **Supplementary Table 1D**). Lipid peroxidation was higher in the pectoral muscle of WR birds [$F_{(1,16)} = 6.98$; $p = 0.018$, **Figure 5A**], while heart [$F_{(1,16)} = 2.44$; $p = 0.138$], liver [$F_{(1,16)} = 2.69$; $p = 0.127$], plasma [$F_{(1,16)} = 0.02$; $p = 0.870$], and erythrocytes [$F_{(1,16)} = 1.73$; $p = 0.207$] showed no significant differences between experimental groups. No significant differences in hydrogen peroxide concentration were observed between treatments in pectoral muscle [$F_{(1,16)} = 0.61$; $p = 0.446$], heart [$F_{(1,16)} = 0.69$; $p = 0.419$], liver [$F_{(1,16)} = 1.03$; $p = 0.330$], or blood plasma [$F_{(1,16)} = 0.31$; $p = 0.586$] (**Figure 5B**). TAC did not differ between treatments for pectoral muscle [$F_{(1,16)} = 1.31$; $p = 0.259$], heart [$F_{(1,16)} = 2.43$; $p = 0.139$], or blood plasma [$F_{(1,16)} = 0.24$; $p = 0.630$; **Figure 5C**]. Pectoral muscle of WR birds had lower glutathione concentrations for both the reduced GSH and oxidized GSSG forms in comparison to the hydrated group (see **Supplementary Table 1D**), but this difference disappeared when body mass was accounted for [$F_{(1,15)} = 0.17$; $p = 0.685$ and $F_{(1,16)} = 0.34$; $p = 0.568$, respectively]. GSH/GSSG ratio in pectoral showed no between treatments [$F_{(1,16)} = 1.14$; $p = 0.302$]. GSH [$F_{(1,16)} = 3.52$; $p = 0.080$], GSSG [$F_{(1,16)} = 0.93$; $p = 0.350$], and GSH/GSSG [$F_{(1,16)} = 2.43$; $p = 0.138$] in liver did not differ between treatments (**Figure 6**).

DISCUSSION

Energy and Osmoregulatory Adjustments

Some bird species respond to (fresh)water limitation by increasing their rates of energy expenditure (including

BMR), a response that is commonly associated with increased osmoregulatory demands in birds inhabiting arid environments or that regularly drink salty water (Gutiérrez et al., 2011; Sabat et al., 2017). An alternative perspective is that such responses could be adaptive, considering that increases in metabolic rate may result in increased MWP, which reduces the need for water conservation and/or reliance on (pre-formed) drinking/food water. To what extent an increase in metabolic rate is explained by the need to produce greater amounts of metabolic water is an open question. Recent research that quantified water budgets in field and laboratory animals have suggested that both extrinsic (e.g., acclimation temperature) and intrinsic (body mass, torpor) affect rates of energy expenditure, which can in turn increase the proportion of metabolic water in the total body water pool (Cooper et al., 2019; Whiteman et al., 2019). Interestingly, seabirds that routinely drink seawater appear to increase their metabolic rate by a greater amount than is required to meet the thermodynamically derived estimates of osmotic work. Such excess increases in metabolic rate could be an adaptive mechanism for increased MWP to maintain water balance (Peña-Villalobos et al., 2013; Sabat et al., 2017).

Dehydration and the associated decrease in body mass (**Figure 1**) is correlated with higher mass-adjusted BMR and MWP in sparrows (**Table 3**). When coupled with reduced TEWL as body mass decreased (**Figure 3B**), higher MWP likely minimized dehydration in the WR treatment. The observed body mass loss and increases in mass-adjusted BMR may suggest that *Z. capensis* may simultaneously increase protein catabolism and production of metabolic water through oxidation (Gerson and Guglielmo, 2011). This may minimize the level of tissue dehydration as most tissues had similar masses regardless of hydration treatment (**Table 1**). While these adjustments could be adaptive and lead to greater resilience to limited water availability, this strategy is costly in terms of mass loss. In birds, catabolism of protein tissue delivers a higher net amount of pre-formed water per gram in comparison to catabolism of lipids (Gerson and Guglielmo, 2011), so the ability to preferentially use protein over lipid reserves may help maintain water balance at the expense of losing muscle and therefore lean body mass. Whole-animal metabolic rates were similar between experimental treatments (**Table 3**) despite significant decreases in body mass observed in the WR group. It is possible that if the observed decrease in body mass was mostly due to water loss rather than to loss of lean mass, then we are over-estimating mass-adjusted BMR because body water does not directly contribute to metabolism. Unfortunately, we were not able to measure changes in body composition before and after the initial experiment, but the second acclimation trial allowed us to assess whether mass loss in WR treatment was driven by changes in lean mass,

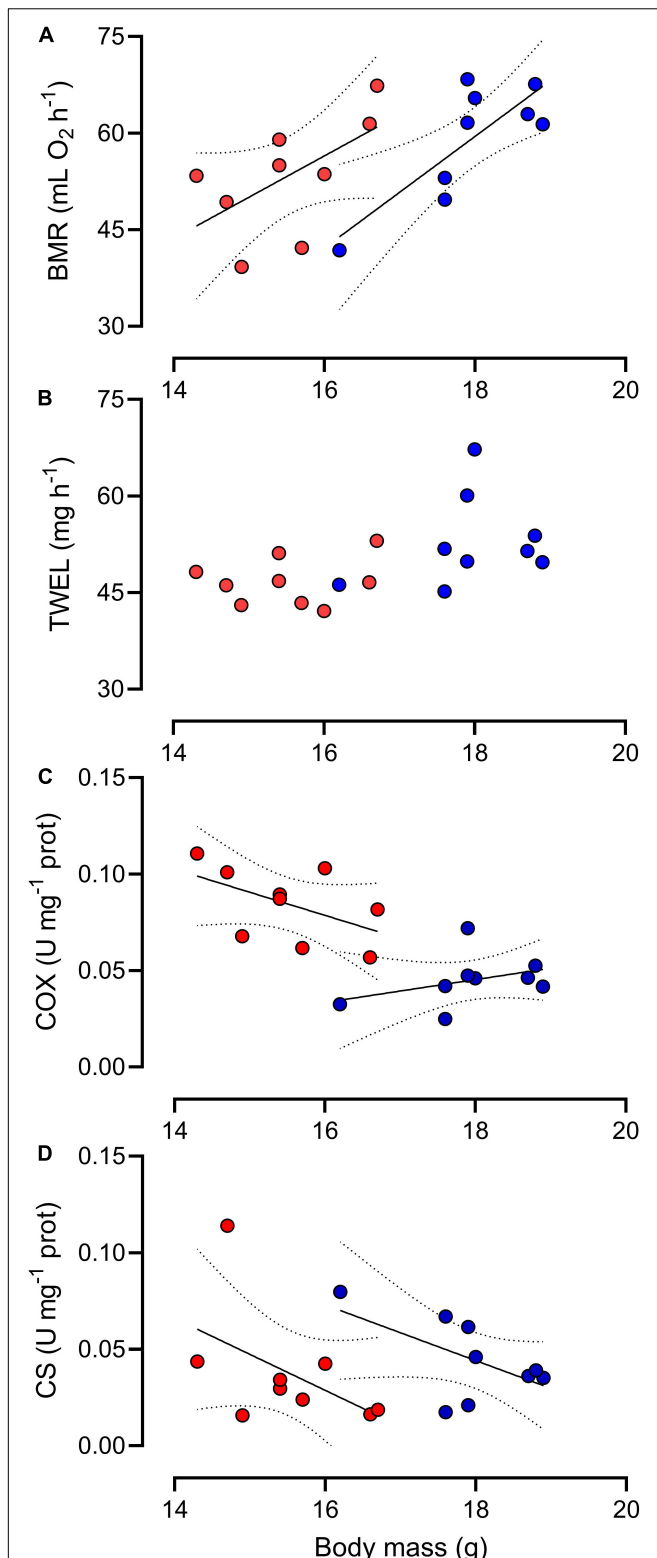


FIGURE 3 | Basal metabolic rate (A), total evaporative water loss (B), cytochrome c-oxidase (C), and citrate synthase (D) activities in erythrocytes as a function of body mass in *Z. capensis* individuals acclimated to control (blue symbol) and water restriction (red symbol) conditions. Lines indicate significant differences between hydrated and dehydrated groups.

fat, or water content. The minimal (2%) difference in hydration index we observed between treatments (ca 0.85, **Table 2**) coupled with the observation that changes in total water content were associated with a decrease in lean mass, suggests that the observed decrease in total water was likely the consequence of lean mass loss (Guglielmo et al., 2011). Indeed, our results suggest that WR birds increased protein catabolism because their lean mass decreased by ~10%, which is consistent with the overall decrease in body mass of ~12%. Thus, while the lower water content in bird tissues may partially explain the higher mass-adjusted BMR in WR birds, most of the change in BMR is likely driven by increases in the specific metabolic activity of internal organs (heart and kidneys) and muscle tissue. Because protein was differentially catabolized between the two experimental groups but whole-animal BMR did not vary, observed changes in mass-specific BMR suggest that the metabolic activity of specific tissues are quite plastic (see below). Nonetheless, we caution that BMR and body condition was measured in different individuals, so this hypotheses is preliminary and required further study.

Variation in avian metabolic rate is usually associated with changes in specific energy expenditure of internal organs, which in turn is influenced by both organ mass and mass-specific metabolism of tissues controlled by mitochondrial density and/or enzyme activity (Vézina and Williams, 2005; Sabat et al., 2019). Results for *Z. capensis* support the idea that dehydration reduces body size which leads to an increase in the relative size of the kidneys and heart (**Table 1**). It appears that an increase in osmoregulatory demand associated with the need to concentrate urine in some passerine species that consume protein-rich and salty resources elicit the same response in kidney and heart morphology (e.g., Sabat et al., 2004a,b; Peña-Villalobos et al., 2013). This is interesting as it has been reported that these internal organs have disproportionately high mass-specific metabolic rates and that the variation in mass-independent metabolism reflects the relative size of this metabolically active machinery (Daan et al., 1990). Unfortunately, we only measured the mass-specific metabolic rate of blood, which was significantly higher in the WR than CT group. Even though blood volume represents ~8–10% of the total volume in birds (Sturkie and Griminger, 1986), it is unknown what proportional contribution blood cells make to the total metabolic rate. Nord et al. (2021) reported that coal tits (*Periparus ater*) and great tits (*Parus major*) increased their mitochondrial respiration rates and CS activity of RBCs in winter, which may be a response to changes in metabolic demands between seasons. In the following sections, we offer alternative explanations for the observed increases in mass-adjusted BMR and COX activity in RBCs and why it could be associated to the costs of osmotic stress.

Changes in Erythrocyte Metabolic Activity

Perhaps the most remarkable result to emerge from our study is that birds exhibited an increase in COX activity in erythrocytes when exposed to chronic dehydration compared to those with *ad lib* access to water. Furthermore, variation in erythrocyte COX activity was associated with variation in levels of energy

TABLE 3 | Mean ($\pm$ SE) and mass-adjusted (least square means, LSM) basal metabolic rate (BMR, $\text{mLO}_2 \text{ h}^{-1}$) and total evaporative water loss (TEWL, $\text{mg H}_2\text{O h}^{-1}$), estimated metabolic water production (MWP, $\text{mg H}_2\text{O h}^{-1}$), the ratio MWP/TEWL and body mass (Mb) of *Z. capensis* acclimated to water restriction (WR) and control (CT) conditions.

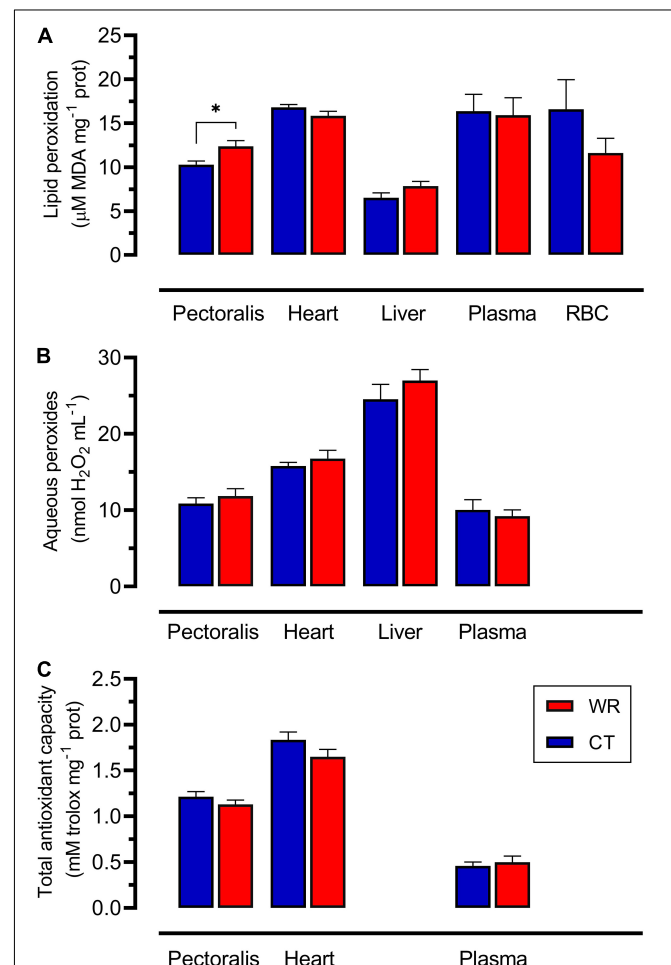
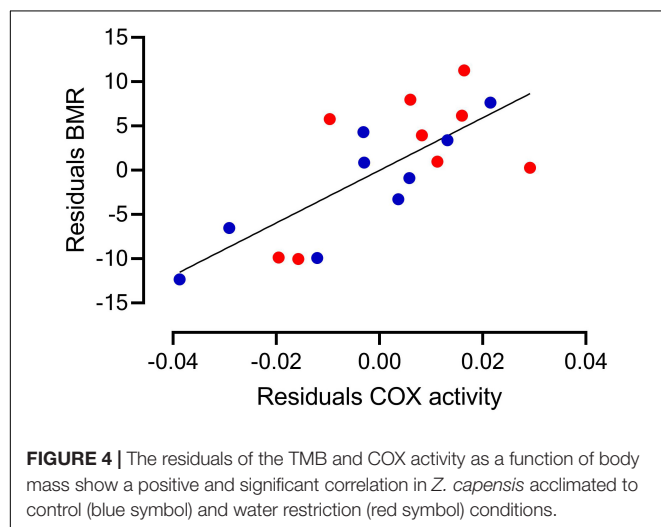
	BMR		TEWL		MWP	MPW/TEWL	mb (g)
	Mean	LSM	Mean	LSM	Mean	Mean	Mean
CT	59.1 ± 3.0	$49.8 \pm 3.3^*$	$52.82 \pm 2.2^*$	50.9 ± 2.2	33.5 ± 5.0	0.63 ± 0.07	$17.9 \pm 2.7^*$
WR	53.3 ± 2.9	62.6 ± 3.3	46.73 ± 1.2	48.61 ± 2.2	30.2 ± 5.1	0.64 ± 0.08	15.2 ± 2.7

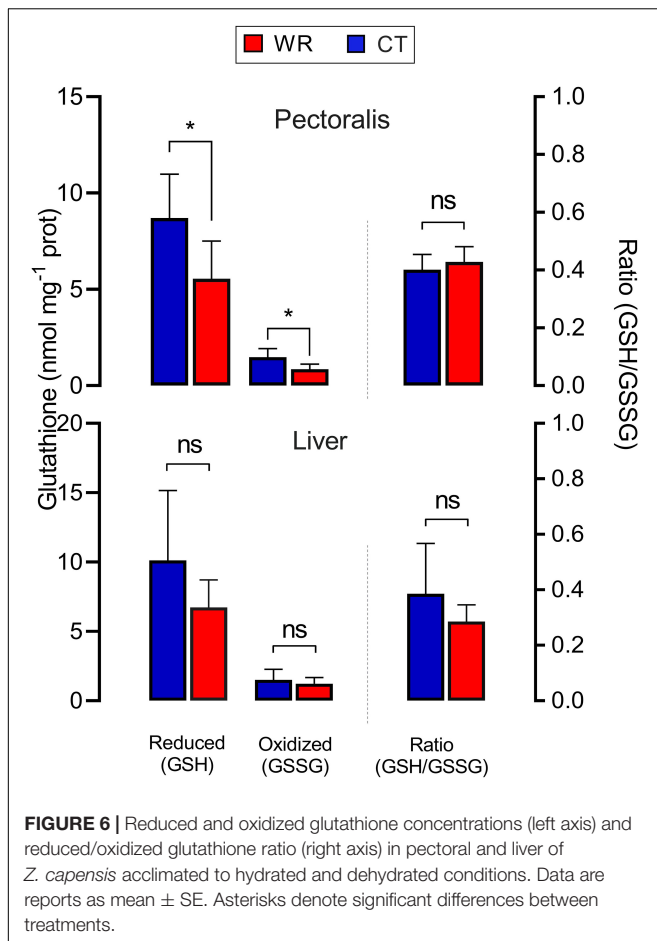
Asterisk denote significant differences between experimental groups. See text for statistics.

expenditure. One of the unique characteristics of birds and often overlooked by ecological and evolutionary physiologists is the presence of nucleated RBCs with functional mitochondria. This key feature has recently caught the attention of physiologists and the existence of intra and interspecific variation in its function has been observed in a few species (Stier et al., 2013, 2015, 2017; Nord et al., 2021). Decades ago, some suggested that ATP production in erythrocytes is involved in the maintenance of ion balance (Na and K) between erythrocytes and plasma (Hunter and Hunter, 1957). Others proposed that the presence of nucleated RBCs with functional mitochondria could be associated with regulatory mechanisms of cell volume using ion transport proteins (e.g., Na^+/H^+ , $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransport and Na^+ channels), which are mediated by ATP-dependent membrane proteins (e.g., NHEs, KCC, and NKCC; Hoffmann et al., 2009). However, our results for *Z. capensis* do not necessarily support the hypothesis that the increase in mitochondrial oxidative activity in the erythrocyte is exclusively driven by the regulation of cell volume. Plasma osmolality was similar in WR and CT birds, and by extension the osmotic pressure experienced by erythrocytes was probably similar between treatments, which also suggests that *Zonotrichia* was able to maintain hydric homeostasis in our experiment.

Although the lack of published data precludes a clear explanation for the observed variation in erythrocyte COX and CS activities, it is interesting that after removing the effect of body mass, only COX activity correlates positively with BMR. This

finding is consistent with those recently reported by Gutiérrez et al. (2019) for the Hudsonian godwit (*Limosa haemastica*), who suggested that the rate of energy expenditure in godwits could be driven by metabolic demands of organs and tissues, including blood. While our results show somewhat contradictory patterns of COX and CS expression in RBCs, comparable results were also observed in Hudsonian godwits collected in winter (Gutiérrez





et al., 2019). As these authors pointed out there could be other physiological processes, such as tissue growth and repair, that differentially modulate the expression of CS and COX in RBCs of birds (Velando et al., 2019).

For *Z. capensis*, at least two non-exclusive alternatives may explain the relationship found between mass-adjusted BMR and erythrocyte COX activity. First, the increase in catabolic capacity in erythrocytes could be a response to vasoconstriction caused by water restriction. It is well known that dehydration causes vasoconstriction of blood capillaries, which decreases the delivery of oxygen to tissues (Kreimeier, 2000). In addition, erythrocytes can detect oxygen tension via microcirculation, regulating the vascular tone of capillaries in specific tissues (Jensen, 2009). In the case of hypoxia caused by vasoconstriction induced to dehydration, erythrocytes could increase the release of vasodilator compounds (e.g., NO, ATP), locally promoting an increase in blood perfusion of tissues that require oxygen (Ellsworth et al., 1995) and thus increasing its oxidative activity. Second, it is likely that changes in metabolic activity in the blood could be driven by increased tissue metabolism. Nutritional sensors, or proteins that respond to fluctuations in nutrients, such as Nicotinamide phosphoribosyltransferase, Sirtuins (silent information regulator), and target of rapamycin complex are likely influenced by the energy status of the organism. When

faced with high oxygen requirements, animals respond by increasing nutrient use by tissues that have higher mass-specific metabolic rates such as the kidney (Gaur et al., 2017; Lee and Yang, 2017), which likely influences other tissues. Thus, the observed relationship between BMR and COX in RBCs could reflect a synergistic response to maintain vascular and respiratory homeostasis, either because the blood cells contribute to blood flow regulating vascular tone, and/or that they are influenced by the same regulatory pathways that reflect the requirements of other energy demanding tissues (e.g., liver). Malkoc et al. (2021) reported that oxygen consumption at the cellular level in RBCs of great tits (*Parus major*) is directly related to oxygen consumption at the organismal level. This pattern was observed only when birds did not display signs of stress as inferred from plasma corticosterone concentrations, indicating that work examining the effects of stress on metabolic rate at both the mitochondrial and organismal level warrants further exploration.

Oxidative Status

Sparrows in the WR group had higher levels of lipid peroxidation as measured by MDA concentrations in pectoral muscle, a sign of higher oxidative damage relative to birds with *ad lib* access to water. These findings are supported by our previous work showing a 400% increase in MDA concentration in *Z. capensis* fed saltwater (Sabat et al., 2017). Observed differences in oxidative damage of pectoral muscle between treatments in this experiment suggest signs of oxidative stress in *Z. capensis* exposed to water restriction, despite pro-oxidants (peroxides) nor antioxidant capacity (TAC) not varying between treatments. Membrane damage in pectoral muscle of dehydrated birds could be explained in part by the observed increase in mass-specific BMR, which might promote the production of free radicals or ROS. Studies have reported contrasting results regarding the functional association between metabolic rate and ROS production under controlled conditions both in ectotherms and endotherms (Hermes-Lima and Storey, 1998; Brzęk et al., 2014; Salin et al., 2015; Zhang and Wong, 2021). Unfortunately, few studies have assessed the effects of dehydration and stress (including oxidative damage) in birds, although a recent publication reported an association between dehydration and increased corticosterone concentrations in free-ranging house sparrows (*Passer domesticus*; Brischoux et al., 2020). While it is known that corticosterone and other glucocorticoids disturb cellular oxidative homeostasis (Costantini et al., 2011; Queisser et al., 2011), it is unknown whether dehydration causes oxidative damage in birds. Further experimental data are required to accurately assess how dehydration affects oxidative status in birds and which intrinsic (e.g., biochemical) and/or extrinsic (e.g., ecological) mechanisms underpin their response.

CONCLUSION

We found that captive *Z. capensis* responds to water restriction by losing mass and increasing their mass-specific maintenance metabolism, a pattern that suggests that the simultaneous

increase in protein catabolism and production of metabolic water through oxidation may help maintain water balance. A noteworthy result is that birds exhibited an increase in COX activity in erythrocytes in response to chronic water restriction, and that variation in COX activity was positively correlated with mass-adjusted BMR. Finally, we found a moderate effect of water restriction on membrane damage of skeletal muscle, which confirms that hydration state and changes in osmotic conditions may cause oxidative damage. Further investigation of how birds use physiological mechanisms to maintain water balance is needed to better understand how they will respond to rapidly shifting environmental conditions that will likely generate increases in ambient temperature but decreases in water availability in aridland ecosystems throughout the world.

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 Institutional Animal Care Committee of the University of Chile.

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AUTHOR CONTRIBUTIONS

PS, SN, FB, and RN designed research. LN, IP-V, CC-R, and JS-H performed research. LN and PS analyzed data. PS, SN, FB, RN, JS-H, LN, and IP-V wrote the manuscript. 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.2021.767280/full#supplementary-material>

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Heterothermy in a Small Passerine: Eastern Yellow Robins Use Nocturnal Torpor in Winter

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Torpor is a controlled reduction of metabolism and body temperature, and its appropriate use allows small birds to adapt to and survive challenging conditions. However, despite its great energy conservation potential, torpor use by passerine birds is understudied although they are small and comprise over half of extant bird species. Here, we first determined whether a free-living, small ~20 g Australian passerine, the eastern yellow robin (*Eopsaltria australis*), expresses torpor by measuring skin temperature (T_s) as a proxy for body temperature. Second, we tested if skin temperature fluctuated in relation to ambient temperature (T_a). We found that the T_s of eastern yellow robins fluctuated during winter by $9.1 \pm 3.9^\circ\text{C}$ on average (average minimum T_s $30.1 \pm 2.3^\circ\text{C}$), providing the first evidence of torpor expression in this species. Daily minimum T_s decreased with T_a , reducing the estimated metabolic rate by as much as 32%. We hope that our results will encourage further studies to expand our knowledge on the use of torpor in wild passerines. The implications of such studies are important because species with highly flexible energy requirements may have an advantage over strict homeotherms during the current increasing frequency of extreme and unpredictable weather events, driven by changing climate.

Keywords: torpor, thermoregulation, passerines, metabolism, climate change, heterothermy, geographical variation, yellow robin

INTRODUCTION

Endotherms can maintain a high body temperature (T_b) across a range of ambient temperatures (T_a) via appropriate adjustment of internal heat production. However, the energetic costs to thermoregulate outside of the thermal neutral zone can be expensive (McKechnie and Lovegrove, 2002; Angilletta et al., 2010). To deal with these energetic costs, many endothermic species use torpor, a controlled reduction in metabolism and typically T_b (Namekata and Geiser, 2009; Ruf and Geiser, 2015). Torpor is used as a strategy to overcome energetically challenging periods such as cold (Maddocks and Geiser, 2007; Romano et al., 2019; Wolf et al., 2020), reduced food/water or foraging opportunities (Nicol and Andersen, 1996; Nord et al., 2009; Smit et al., 2011) or even hot conditions (Reher and Dausmann, 2021), and its use varies considerably among avian taxa (Geiser, 2021). Some evidence of intra-specific variation in torpor patterns along latitudinal or elevation gradients (Geiser and Ferguson, 2001; Dunbar and Brigham, 2010; Zervanos et al., 2010; Stawski and Geiser, 2011) suggests that T_a may play a major role in torpor expression in passerines, but only

little information on geographical variation is available for birds (Chaplin, 1976; Sharbaugh, 2001). Although some potential costs may occur at low T_b s on a cellular level (Nowack et al., 2019), the use of torpor may reduce predation risk by increasing antipredator behavior (but see Amo et al., 2011; Turbill and Stojanovski, 2018), and in small diurnal birds can reduce metabolic demands by as much as 50% (Cooper and Gessaman, 2005) and may increase survival by 58% (Brodin et al., 2017).

The use of torpor for energy conservation is common in a range of small mammals (e.g., Ruf and Geiser, 2015; Nowack et al., 2020) and in several non-passerine bird species such as hummingbirds and nightjar relatives (Hiebert, 1992; Körtner et al., 2000; Shankar et al., 2020). In contrast, the extent of the use of torpor by passerines, a group which includes over half of extant birds species, is understudied (McKechnie and Lovegrove, 2002). Despite evidence for large nocturnal T_b reduction dating back to the late 1950's [e.g., in Redpoll *Carduelis flammea* and tree sparrow *Passer montanus*, Steen (1958)], and further anecdotal evidence of nocturnal torpor in multiple species in the 1970's [Crimson chat (*Epthianura tricolor*), red-capped robin (*Petroica goodenovii*), white-fronted honeyeater (*Phylidonyris albifrons*) and banded whiteface (*Aphelocephala nigricincta*); Ives (1973), white-backed swallow (*Cheramoeca leucosternum*); Serventy (1970)], little progress has been made in the field. Some small passerine species have been reported to use torpor under controlled conditions: Golden-collared manakins [*Manacus vitellinus*; Bucher and Worthington (1982)], dusky woodswallows [*Artamus cyanopterus*; Maddocks and Geiser (2007)], rifleman [*Acanthisitta chloris*; McNab and Weston (2018)], and malachite sunbirds [*Nectarina famosa*; Downs and Brown (2002)] dropped their T_b to 27–30°C at T_a s of 19.5, 6, 11, and 5°C, respectively. The rifleman has also been reported to decrease metabolic rate to as low as 21% of its expected basal metabolic rate (McNab and Weston, 2018). Torpor under controlled environment has also been reported in species adapted to very cold conditions. The black-capped chickadees (*Poecile atricapilla*) from Alaska reduced T_b by about 8°C in both winter and summer when exposed to T_a –30°C, and actively rewarmed (Sharbaugh, 2001). Metabolic rate reduction has also been observed in scarlet-backed Flowerpecker (*Dicaeum cruentatum*), which reduced metabolic rates by over 70% during torpor vs. non-torpor state (Bushuev et al., 2021). While this evidence supports torpor use in passerine species, it is important to evaluate these functions in the wild. Captive conditions do not represent the complexity of the thermal conditions and ecological complexity (e.g., food abundance, predation risk) experienced in the wild, and laboratory studies may therefore underestimate the use of torpor by wild animals (Geiser et al., 2000; O'Connor et al., 2017).

To date, only two passerine species studied in the wild have been shown to express torpor. The T_b of noisy miners (*Manorina melanocephala*) fell by 7°C on average, to a minimum of 33°C during winter in eastern Australia, where night time temperatures frequently drop to near 0°C (Geiser, 2019), and fairy wrens (*Malurus cyaneus*) dropped their skin temperature (T_s) by over 14.5°C, with minimum T_s of 27.4°C recorded at average minimum T_a of about 3°C (Romano et al., 2019). Other

passerines species which were studied in the wild remained euthermic, even during challenging conditions [e.g., red-headed finch *Amadina erythrocephala* (McKechnie and Lovegrove, 2003), five species of tropical montane passerines (Burnett et al., 2019), willow tit *Parus montanus* (Reinertsen and Haftorn, 1984), bronze mannikins *Spermestes cucullatus* (Lovegrove and Smith, 2003), great tits *Parus major* (Nilsson et al., 2020), blue tits *Cyanistes caeruleus* (Nord et al., 2009)]. The lack of extensive studies on torpor use in wild passerines is somewhat surprising, because although evidence is limited, data in the wild and under controlled environment do suggests that some passerine species are capable of large T_b reduction. Moreover, many passerine species are insectivorous, small and diurnal and cannot feed at night when T_a s are low. The abundance of insects and other food also typically decreases in winter, therefore, it is likely that many passerine species could benefit greatly from the use of torpor.

The aim of our study was to examine the thermal energetics of a small passerine species, the eastern yellow robin (*Eopsaltria australis*; hereafter “eastern robin”), during winter at a cool temperate climate site in the eastern Australian Northern Tablelands (elevation range 980–1050 m). We determined whether individuals express torpor by measuring the magnitude of T_s reduction and whether this reduction is related with ambient temperature (T_a). Additionally, we discuss our results in comparison with the thermal energetics of a closely related species, the western yellow robin (*Eopsaltria griseogularis*) (hereafter “western robin”), which is the only other member of the genus *Eopsaltria* in Australia (Loynes et al., 2009). The western robin was studied in a Mediterranean climate in WA (Douglas, 2017).

MATERIALS AND METHODS

We captured four adult wild-living eastern robins (2 male, 2 female; mean body mass 19.4 g) during the southern hemispheric winter (July–August) in Imbota Nature Reserve, NSW, Australia (30.58°S, 151.72°E), a 218 ha open *Eucalyptus* and *Acacia* woodland. The population of eastern yellow robins in Imbota included on average nine pairs between 2000 and 2003, but the population likely declined (Debus, 2006) as a result of low reproduction in this region, mainly due to nest predation by Pied Currawongs (*Strepera graculina*) (Debus and Ford, 2012), and eastern robins produce too few independent young to replace adult mortality (Zanette, 2000; Debus, 2006). Additionally, eastern robins appear to be most sensitive to loss, fragmentation and degradation of habitat in rural landscapes (Watson et al., 2001, 2002; Lambeck, 2002), and an intense drought from mid-2017 to 2020 and major fire events in summer 2019 in NSW may deteriorated the quality of the site and caused further population decline. Indeed, during our fieldwork we could not find more than five individual eastern robins on the site.

Average daily minimum and maximum T_a s for Imbota in midwinter (July) are 1.3 and 12.2°C, and average summer T_a reach up to 26.3°C (Bureau of Meteorology, 2021). After capture, birds were held in a cotton bag until processed. The birds were weighed to the nearest g with a digital scale (HCB-1002,

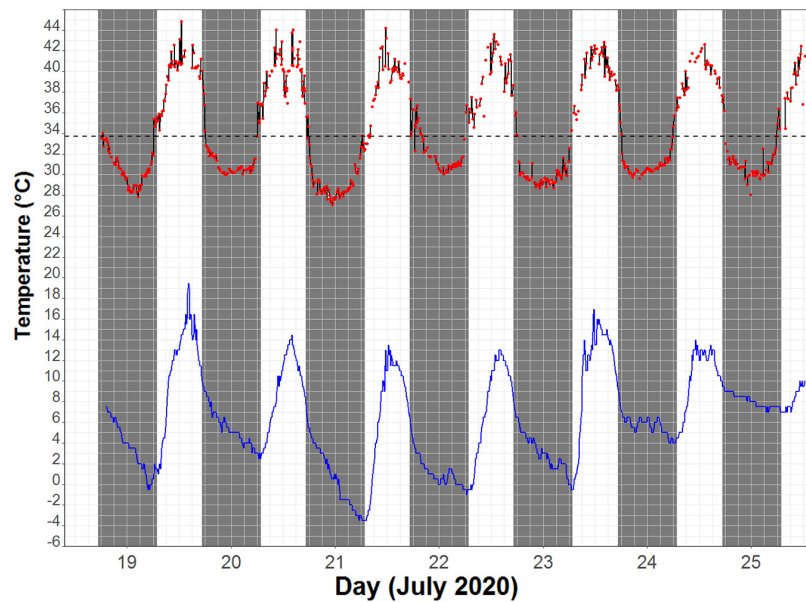


FIGURE 1 | An example of skin temperature (T_s) fluctuation of one individual (red line) and the corresponding ambient temperature (T_a) measured near the roosting site (blue line) over a 7-day period. Black dashed line depicts the torpor threshold (33.7°C), calculated as 5°C below the resting T_s .

Adam highland HCB). Each bird was banded with an individual numbered metal band and a unique combination of color bands. We then attached a temperature-sensitive radio transmitter (LB-2XT, 0.33 g, Holohil Systems Ltd., Canada) directly to the skin, between the shoulder blades using a latex-based adhesive (12% resin, Manfred Sauter GmbH). Transmitters were calibrated in a water bath to the nearest 0.1°C between 25.0 and 40.0°C in $\sim 5.0^\circ\text{C}$ increments with a precision digital thermometer (Model 15-077-8, Fisherbrand, United States) before attachment. T_s of each individual bird was calculated from the interval between two pulses following the calibration curve ($R^2 > 0.98$), and was recorded automatically in 10-min intervals with receiver/loggers fitted with an H-frame antenna (Titley Electronics or Telonics Inc., ARI, United States) placed near the roost site. Transmitters remained attached to the birds for 7–15 days, and we recorded T_s over a total of 30 days. Birds were active during the day and mostly remained within range of the logger. We excluded from the analysis daytime data for one bird, which often was beyond range during the day. We also excluded nights where signal was lost (as a result of bird moving or signal interrupted), which resulted in a total of 7 measurement nights for 3 individuals and 6 measurement nights for 1 individual and a total of 2678 T_s data points.

Air temperature (T_a) was recorded every 10 min using four temperature loggers (iButton DS1922L, 0.06°C resolution, Maxim Integrated Products, Inc., Sunnyvale, CA, United States) placed on the southern (shady) side of trees near the roosting locations. We fitted two separate linear mixed effect models with bird identity as a random effect to explain (1) minimum T_s as a function of body mass and mean T_a and (2) minimum T_s as a function of body mass and minimum T_a as explanatory variables. Second, we also ran a linear mixed effect model with average T_s

range (maximum T_s –minimum T_s), again as a function of body mass and mean and minimum T_a in two separate models. The torpor threshold was defined as a reduction by $>5^\circ\text{C}$ below the resting T_s (Schleucher, 2004; Ruf and Geiser, 2015). The resting T_s was calculated as the average minimum T_s during daytime, between 10:00 and 15:00 of two of our studied individuals, which had sufficient daytime measurements to calculate average minimum T_s .

Statistical analysis was conducted using R version 3.6.0 (R Development Core Team).¹ The R-function *lme* in R package *nlme* was used for the mixed effect models (Pinheiro et al., 2020). Data are presented as the mean $\pm$ s.d. of individual mean daily values.

RESULTS

The average daytime T_a (between sunrise and sunset) at our study site for the eastern robin was $9.5 \pm 4.3^\circ\text{C}$ (range -3.5 to 19.5°C) and average night-time T_a was $4.0 \pm 3.1^\circ\text{C}$ (range -3.5 to 10°C). The average maximum and minimum T_a recorded were $14.3 \pm 2.2^\circ\text{C}$ and $1.3 \pm 3.1^\circ\text{C}$, respectively. T_a dropped below 0°C on three days (Figure 1). Rain (21 and 7 mm) occurred only on 2 days (26 and 27 July), and coincide with T_s measurements of only one bird. We therefore did not analyze the effect of rain on torpor use in this study.

The T_s of eastern robins decreased from an average maximum T_s of $39.6 \pm 3.1^\circ\text{C}$ (range 44.8 – 34.0°C), 0.3 ± 0.3 h after sunset (19:10 h), to average minimum T_s of $30.1 \pm 2.3^\circ\text{C}$ (range 26.0 – 34.6°C). The torpor threshold was set at 33.7°C based on average

¹<http://www.R-project.org/>

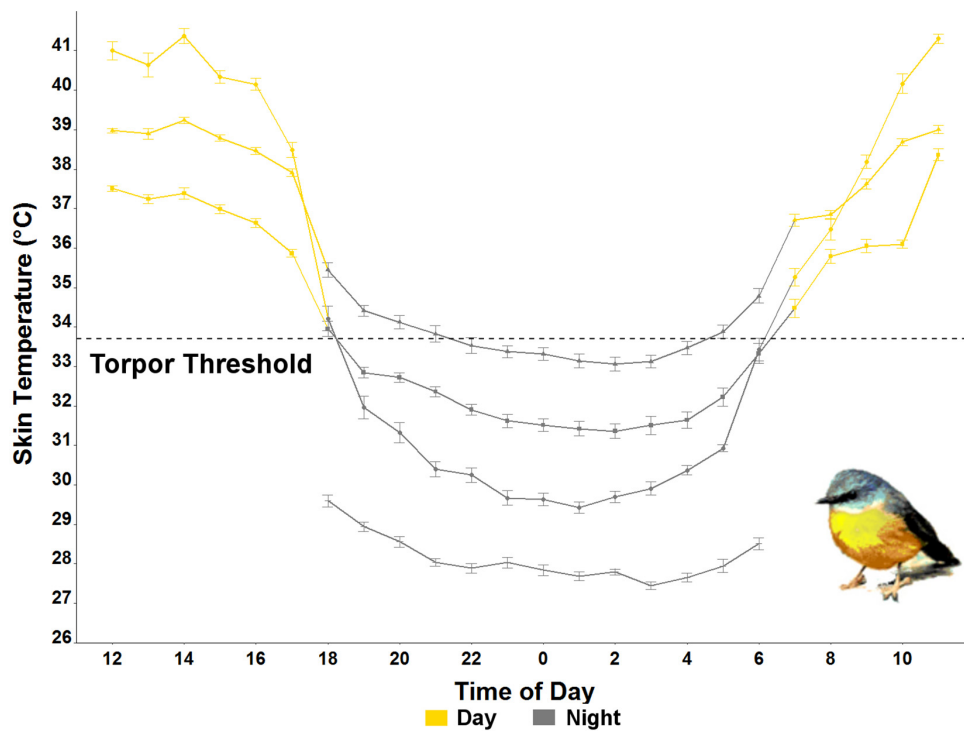


FIGURE 2 | Average hourly skin temperature (T_s) $\pm$ standard error of the four individual eastern yellow-robin measured in July in the wild in NSW, Australia (2020). The torpor threshold (black dashed line at 33.7°C) was calculated as 5°C below the resting T_s .

resting T_b of 38.7°C, which is similar to a rest phase T_b of 38.9°C found previously in birds from the order Passeriformes (Prinzinger et al., 1991). Torpor bout duration (TBD) during which the T_s remained below the torpor threshold lasted for 10.3 h on average, with a maximum individual TBD of 14 h. Rewarming of T_s (the time in the morning where T_s was only followed by higher T_s for at least six consecutive measurements) started -2.6 ± 0.9 h relative to sunrise (06:45 h), when the average T_a was $2.1 \pm 2.9^\circ\text{C}$. Minimum T_s was reached at 00:36 h on average (range between 21:21 and 02:41; **Figure 2**). Average individual daily fluctuations of T_s were $9.1 \pm 3.9^\circ\text{C}$ (range: 3.4–17.1°C), with maximum daily T_s range of 17.1°C recorded on 20 July (11:21 h), when the T_s of one individual dropped to 27.1°C (at a T_a of 0.5°C), the absolute minimum recorded (**Figure 1**).

The average minimum T_s , and average daily range of T_s of the eastern robin were significantly affected by average T_a ($T_s = 28.01 + 0.34T_a$, $df = 22$, $t = 3.18$, $P < 0.01$; $T_{s\text{range}} = 2.37 - 0.05T_a$, $df = 19$, $t = -2.25$, $p < 0.05$, respectively), and by minimum T_a ($T_s = 29.82 + 0.18T_a$, $df = 22$, $t = 2.21$, $P < 0.05$; $T_{s\text{range}} = 2.13 - 0.03T_a$, $df = 19$, $t = -2.30$, $p < 0.05$, respectively), but were independent of body mass at capture ($p > 0.05$).

DISCUSSION

We provide new evidence that the eastern robin, a small ~20 g Australian passerine, uses nocturnal torpor and reduces its night-time T_s during winter to as low as 27.1°C, with TBD lasting over

10 h on average. At the time rewarming began, T_a s were still low, on some days below 0°C, and rewarming commenced almost 3 h before sunrise (**Figure 1**), suggesting endogenously controlled rewarming to prepare for the active phase of morning foraging (Hiebert, 1992).

Although our measurements are based on only four individuals and T_s was used to quantify their thermal biology, we contend that our data are meaningful. All individuals showed a similar, highly predictable daily T_s change and while there is some gradient between core T_b and T_s (Lovegrove and Smith, 2003), T_s is very close to core T_b in small birds, variation being less than 2°C in the 50 g common poorwill (Brigham, 1992), and not exceeding 4°C even in an 80 g owl (Smit and Mckechnie, 2010). In comparison to a rest phase T_b of 38.9°C in birds from the order Passeriformes (Prinzinger et al., 1991), T_s reduction in our study is on average 8.7°C, with maximum reduction of 12.9°C. Moreover, if T_a was influencing the measurement T_s significantly, we would expect to see T_s fluctuate closely with T_a . Instead, T_s started to increase when T_a was still low (about 2°C on average) and even falling (**Figure 1**). Therefore, even with a small error from the T_s measurements, the data clearly show that the birds used nocturnal torpor.

The magnitude of nocturnal T_s drop during torpor increased with decreasing mean and minimum T_a . This relationship was previously found in other passerine species (Reinertsen and Haftorn, 1983; Nord et al., 2009, 2011; Romano et al., 2019) and supports the assumption that the controlled reduction of T_s aims to conserving energy during energetically expensive periods,

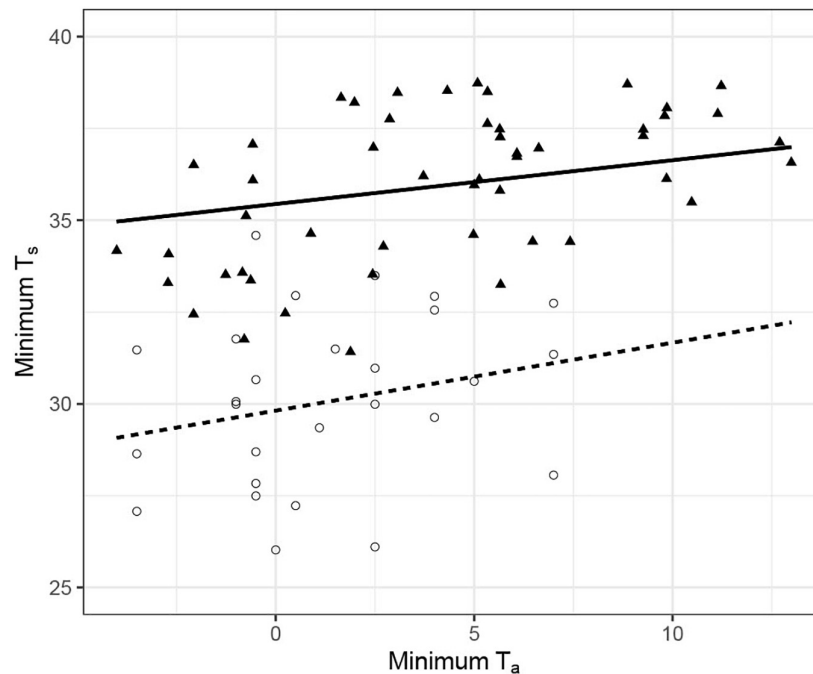


FIGURE 3 | The relationship between minimum skin temperature (T_s ; °C) and ambient temperature (T_a ; °C) in the eastern ($N = 4$; open circles, dashed line) and western ($N = 7$; filled triangles, solid line) yellow robin. Lines were fitted with a linear mixed effect model with minimum T_s of robins from east and west population separately, in relation to minimum T_a and bird identity as a random effect. Data for the western yellow robin was extracted from Douglas (2017). Like eastern robins (details of statistics in the text), the minimum T_s of the western robins were significantly affected by minimum T_a ($T_s = 35.44 + 0.11T_a$, $df = 40$, $t = 2.86$, $P < 0.01$).

such as high energy expenditure required for thermoregulation. A reduction of energy expenditure by 10–20% was demonstrated in several captive avian species when exposed to T_a 0–10°C, with T_b reduction of 3–11°C [mountain chickadees *Poecile atricapillus*, and juniper titmice *Baeolophus ridgwayi* (Cooper and Gessaman, 2005); willow tit *Parus montanus* (Reinertsen and Haftorn, 1984)]. Captive noisy miners *Manorina melanocephala* reduced metabolic rate by ~40% when they were measured under T_a s of 0–15°C, with a T_b drop of only 4°C (Geiser, 2019). To calculate the predicted metabolic reduction in our studied eastern robins, we followed the equation for metabolic rate $M = C' (T_b - T_a)$ (Snyder and Nestler, 1990), where M = metabolic rate and C' = thermal conductance. C was set as 0.186 following Douglas (2017) for western robin, a closely related species of similar size as the eastern robin, because our birds were in steady-state torpor and thermal conductance under such conditions is strongly related to body mass (Schleucher and Withers, 2001; Geiser, 2004) and is often steady below TNZ over a wide range of T_a [but may deviate under extreme conditions; Fristoe et al. (2015)]. Based on these calculations, we estimated that the metabolic rate of a 20 g eastern robin during torpor was about 4.9 ml O_2 $g^{-1} h^{-1}$ at $T_a = 0.5^\circ C$ compared to predicted metabolic rate of over 7 ml O_2 $g^{-1} h^{-1}$ at the same T_a if the bird remained euthermic. Our calculated MR of torpid birds is still above BMR of eastern robin (3.28 ml O_2 $g^{-1} h^{-1}$; Bech et al., 2016), but this is often the case during nocturnal torpor in small passerines (e.g., Maddocks and Geiser, 2000). Importantly, the calculation predicts that the eastern robin reduced MR during torpor by

about 24% derived from the average minimum T_s reduction, compared to its rest-phase T_s of 38.7°C. This prediction is appropriate here because eastern robins maintained a $T_b - T_a$ differential of $> 25^\circ C$ via thermoregulation during torpor, unlike many torpid mammals, which when thermoconforming, can have $T_b - T_a$ differentials of often $< 1^\circ C$ (Geiser, 2021). When the minimum recorded T_s of 27.1°C was used for the calculations, the MR was reduced by about 32%.

Our data suggest torpor use by eastern robins as an energy saving strategy. Although our study only report data from four individuals, it clearly demonstrates that eastern robins are capable of such energy savings through thermal flexibility. However, we do not argue that our results apply to the whole species. Over its entire range, local conditions are key elements in driving physiological strategies, and we therefore expect some variance in thermal energetics among geographically separated species and/or populations. Indeed, the western robin, a closely related species to the eastern robin (mean body mass 18.5 g), and the only other member of the genus *Eopsaltria* in Australia (Loynes et al., 2009), remained euthermic in Dryandra woodland National Park (mean T_s 35°C), with a Mediterranean climate, lower elevation site (228 m) in WA during winter (Douglas, 2017). The minimum T_s recorded of the western robins were about 5°C higher on average than that of the eastern robins. The variation in the thermal energetics between the western and eastern robins cannot be explained by the acute exposure to different T_a because night-time T_a in Dryandra dropped similarly to our study site, to a minimum T_a of $-3.95^\circ C$ (Figure 3). The

two robin species are also very similar in external appearance and size, excluding body mass as a potential explanation for the observed differences in thermal biology. The climate in Dryandra is mild relative to Imbota (i.e., winter T_a rarely drop below 0°C; Bureau of Meteorology, 2021). Therefore, a potential explanation for these differences may be that long-term thermal adaptation to the conditions in the occupied habitat may drive geographical variation in physiological traits, likely to minimize the potential costs accompanying the use of torpor. Similar differences have been observed between captive bred and wild-caught feathertail gliders (Geiser and Ferguson, 2001) and between captive woodchucks originate from different populations (Fenn et al., 2009).

Although the western robins did not express torpor like the eastern robins in our study, we do not argue that western robins, or any other passerine species which were studied in the wild and did not express torpor, are not capable of some form of heterothermy to conserve energy. Scarlet-backed flowerpeckers *Dicaeum cruentatum*, for example, which were studied in captivity for 3 years, appear to express torpor only in years with poor food quality (Bushuev et al., 2021). Other species have been considered homeothermic for decades until they were studied at the relevant conditions that trigger the use of torpor [e.g., mouse lemurs *Microcebus murinus* and *M. myoxinus*; (Ortmann et al., 1997)] and there are many other examples. Therefore, western robins, although they may not use torpor routinely as a means to balance their daily energy budget, still may express torpor under challenging conditions, such as drought or extreme cold, to increase the probability of survival.

Our study provides evidence for torpor expression in a small ~20 g passerine, and suggests plasticity in the expression of torpor, in response to local environment. The resulting flexibility in energy requirements allows animals to optimize energy conservation to increase fitness and has the advantage over strict homeotherms during the current increasing frequency of extreme and unpredictable weather events, driven by changing climate. Indeed, despite range reduction and extreme climatic events, eastern yellow robins manage to survive in a marginal reserve and perhaps torpor expression permits them to overcome these energetically challenging periods and maintain fitness. The question that remains to be answered is whether individuals that previously did not encounter extreme climatic events, or local climatic changes, are able to apply thermal strategies to adjust to new environmental conditions. Resolving these questions is important in light of climatic changes that are expected to change local conditions for populations, but also for translocation

conservation programs (Cooper et al., 2018), which need to consider the physiological limits of introduced species.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the University of New England Animal Ethics Committee (Authority No. AEC20-009).

AUTHOR CONTRIBUTIONS

YA-R and FG formulated the idea and designed the study. YA-R, CB, and JM performed the fieldwork. YA-R performed the data analysis with advice from JM. YA-R wrote the manuscript with advice from all other authors. 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.2021.759726/full#supplementary-material>

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Avian evaporative cooling and the maintenance of body temperature (T_b) below lethal limits during heat exposure has received more attention in small species compared to larger-bodied taxa. Here, we examined thermoregulation at air temperatures (T_{air}) approaching and exceeding normothermic T_b in three larger birds that use gular flutter, thought to provide the basis for pronounced evaporative cooling capacity and heat tolerance. We quantified T_b , evaporative water loss (EWL) and resting metabolic rate (RMR) in the ~170-g Namaqua sandgrouse (*Pterocles namaqua*), ~430-g spotted thick-knee (*Burhinus capensis*) and ~670-g spotted eagle-owl (*Bubo africanus*), using flow-through respirometry and a stepped T_{air} profile with very low chamber humidities. All three species tolerated T_{air} of 56–60°C before the onset of severe hyperthermia, with maximum T_b of 43.2°C, 44.3°C, and 44.2°C in sandgrouse, thick-knees and eagle-owls, respectively. Evaporative scope (i.e., maximum EWL/minimum thermoneutral EWL) was 7.4 in sandgrouse, 12.9 in thick-knees and 7.8 in eagle-owls. The relationship between RMR and T_{air} varied substantially among species: whereas thick-knees and eagle-owls showed clear upper critical limits of thermoneutrality above which RMR increased rapidly and linearly, sandgrouse did not. Maximum evaporative heat loss/metabolic heat production ranged from 2.8 (eagle-owls) to 5.5 (sandgrouse), the latter the highest avian value yet reported. Our data reveal some larger species with gular flutter possess pronounced evaporative cooling capacity and heat tolerance and, when taken together with published data, show thermoregulatory performance varies widely among species larger than 250 g. Our data for Namaqua sandgrouse reveal unexpectedly pronounced variation in the metabolic costs of evaporative cooling within the genus *Pterocles*.

Keywords: Charadriiformes, heat tolerance, hyperthermia, Pteroclitiformes, Strigiformes, thermoregulation

INTRODUCTION

Evaporative cooling and the maintenance of body temperature (T_b) below environmental temperature is critical for terrestrial taxa that encounter operative temperatures (Bakken, 1976; Robinson et al., 1976) exceeding normothermic T_b , or when metabolic heat produced during activity must be dissipated to avoid T_b reaching lethal limits (e.g., Nilsson and Nord, 2018; Thompson et al., 2018). There is increasing evidence that among endotherms, evaporative cooling has evolved in tandem with organismal traits such as surface water dependence (Czenze et al., 2020) and roost microsite preferences (Maloney et al., 1999; Cory Toussaint and McKechnie, 2012; Czenze et al., in press). Gaining a better understanding of the upper limits to animals' evaporative cooling capacity and their exposure to extreme heat events has taken on new urgency in light of rapid global heating (IPCC, 2021), increasingly frequent heat-related mortality events (Welbergen et al., 2008; Ratnayake et al., 2019; McKechnie et al., 2021b) and large increases in risks of lethal dehydration or hyperthermia predicted for coming decades (Albright et al., 2017; Conradie et al., 2020).

On account of birds being predominantly small and diurnal and making limited use of thermally buffered diurnal refugia such as burrows, avian heat tolerance and evaporative cooling capacity has been the subject of long-standing interest among ecological physiologists (e.g., Dawson, 1954; Lasiewski and Seymour, 1972; Weathers, 1981). Maximum evaporative cooling capacity varies substantially among avian orders (reviewed by McKechnie et al., 2021a). Maximum ratios of evaporative heat loss/metabolic heat production (EHL/MHP) are consistently higher in taxa that use pronounced gular flutter (Dawson and Fisher, 1969; O'Connor et al., 2017) or have the capacity for rapid cutaneous evaporative water loss (Calder and Schmidt-Nielsen, 1967; Marder and Arieli, 1988; McKechnie et al., 2016a) compared to groups that rely on panting as their primary pathway of evaporative heat loss (Dawson, 1954; Weathers, 1981). Whereas fractional increases in EWL are broadly consistent across taxa, the metabolic heat production associated with panting means that evaporative cooling capacity and heat tolerance limits tend to be lower in passerines, which represent > 50% of extant birds, compared to some non-passerine orders (McKechnie et al., 2021a).

The number of species for which evaporative cooling capacity and heat tolerance limits have been quantified under standardised conditions has increased substantially in the last decade, facilitating direct comparisons among taxa (e.g., Whitfield et al., 2015; Smith et al., 2017; Smit et al., 2018). However, the body mass (M_b) of most species investigated is <100 g, and much less is known about limits to heat tolerance in larger species. Two notable exceptions include the ~610-g brown-necked raven (*Corvus ruficollis*), in which Marder (1973) reported $T_b = 44.7^\circ\text{C}$ and EHL/MHP = 1.67 at an air temperature (T_{air}) of 50°C , and the ~1300-g MacQueen's bustard (*Chlamydotis macqueenii*) which maintains $T_b < 43^\circ\text{C}$ even at T_{air} as high as 55°C (Tieleman et al., 2002). In the latter study, bustards showed no discernable increase in RMR above a lower critical limit of thermoneutrality of 28.5°C , but a ~10-fold

increase in evaporative water loss (EWL) above minimum thermoneutral levels.

In light of the limited data available for medium- and large-bodied birds and the negative allometric scaling of variables such as surface area – volume ratio and EWL as a fraction of total body water per unit time, we quantified upper limits to heat tolerance and evaporative cooling capacity in three medium-sized southern African species: Namaqua sandgrouse (*Pterocles namaqua*; ~170 g, Pterocliiformes), spotted thick-knee (*Burhinus capensis*; ~430 g, Charadriiformes) and spotted eagle-owl (*Bubo africanus*; ~670 g, Strigiformes). We employed the same standardised methods of a stepped T_{air} profile and very low chamber humidities used in several recent studies (e.g., Whitfield et al., 2015; Smith et al., 2017; Smit et al., 2018). Because these three orders are all known to employ gular flutter (Bartholomew et al., 1968; Thomas and Robin, 1977; Ward, 1990), we expected that maximum EHL/MHP and heat tolerance limits (HTL: maximum T_{air} tolerated before onset of severe hyperthermia) are relatively high in these species.

MATERIALS AND METHODS

Study Site and Species

The thermoregulatory capacity of three species representing three orders was assessed during the 2018–2019 austral summer (2 December – 4 February) at the 76-km² Black Mountain Mine Conservation Area ($29^\circ 18'S$, $18^\circ 51'E$) near Aggeneys, Northern Cape, South Africa. The Black Mountain Mine Conservation Area is situated within the Koa River Valley, which consists predominantly of red sand dunes with scattered shrubs (*Rhigozum trichotomum*) and large-seeded grasses (*Brachiaria glomerata*, *Stipagrostis amabilis*, *Stipagrostis ciliata*, and *Stipagrostis brevifolia*). In parts of the study site, the dunes are separated by unvegetated gravel plains. The climate is arid with mean annual precipitation of ~100 mm and daily maximum T_{air} that ranged from 26.4 to 40.3°C during the study period (Kemp et al., 2020). The study species varied in M_b , as well as foraging behaviour, water dependence and activity phase (Table 1). We captured eight Namaqua sandgrouse (hereafter, sandgrouse), 10 spotted thick-knees (hereafter, thick-knees), and seven spotted eagle-owls (hereafter, eagle-owls). Namaqua sandgrouse were captured at a regularly frequented drinking spot using mist nets. Eagle-owls were captured at night opportunistically using a modified spring trap baited with two live mice. Thick-knees were captured at night using a hand-held net and torch.

Captured birds were placed in clean cloth bags and transported (approximately 45-min trip by vehicle) to an animal holding room where they were kept indoors in individual cages (0.8 m³). Individuals were kept in cages for no more than 12 h and provided with an *ad libitum* supply of water. In the case of the eagle-owls and thick-knees, experimental procedures took place the morning following capture, ≥ 10 h after birds last fed. In the case of sandgrouse, the time between capture and being placed in respirometry chambers was ~3 h.

TABLE 1 | Taxonomy, body mass, activity period, major diet and water dependence of the three study species.

Species	Order/Family	Body mass (g) (sample size) [range]	Activity period	Diet	Drinks?
Namaqua Sandgrouse <i>Pterocles namaqua</i>	Pteroclitiformes/Pteroclitidae	165.8 ± 17.2 (8) [130–185]	Diurnal	Seeds	Yes
Spotted Thick-knee <i>Burhinus capensis</i>	Charadriiformes/Burhinidae	434.3 ± 34.4 (10) [362–484]	Nocturnal	Invertebrates	Yes
Spotted Eagle-Owl <i>Bubo africanus</i>	Strigiformes/Strigidae	667.9 ± 77.1 (7) [570–775]	Nocturnal	Vertebrates/invertebrates	Yes

Body masses were obtained by weighing birds immediately following capture, and activity, diet and drinking behaviour were obtained from species accounts in Hockey et al. (2005).

Air and Body Temperature

A temperature-sensitive passive integrated transponder (PIT) tag (BioTherm13, Biomark, Boise, ID, United States) injected into each individual's abdominal cavity measured T_b . We calibrated these tags in a circulating water bath (model F34; Julabo, Seelbach, Germany) over a 35–50°C range against a thermistor probe (TC-100; Sable Systems, Las Vegas, NV, United States). We placed PIT-tagged individuals in a metabolic chamber placed next to an antenna connected to a transceiver system (HPR+; Biomark). The PIT tags we used for this study were from the same batch as the 23 tags calibrated by Freeman et al. (2020), who found that measured values deviated from actual values by $0.28 \pm 0.23^\circ\text{C}$. We inserted a thermistor probe (TC-100; Sable Systems) sealed with a rubber grommet through the approximate centre of the lid of each metabolic chamber to measure T_{air} during the gas exchange measurements.

Gas Exchange

We measured EWL and carbon dioxide production ($\dot{V}_{\text{CO}_2}$) using an open flow-through respirometry system. The metabolic chamber consisted of either a 12.8-L (sandgrouse) or 60-L (eagle owl and thick-knee) air-tight plastic container (previously shown to not adsorb water vapour; Whitfield et al., 2015). Plastic mesh platforms were placed in the metabolic chambers to ensure individuals were elevated at least ~10 cm above a ~1 cm layer of mineral oil. These plastic chambers were situated within a temperature-controlled custom-built aluminium box (~640 L). The T_{air} within the chamber was controlled via a Peltier device (AC-162 Thermoelectric Air Cooler; TE Technology, Traverse City, MI, United States) connected to a digital controller (TC-36-25-RS485 Temperature Controller; TE Technology) and mounted to the side of the box.

Atmospheric air was supplied by an oil-free compressor and passed through a membrane dryer (Champion CMD3 air dryer and filter; Champion Pneumatic, Princeton, IL, United States) to scrub water vapour before being split into baseline and experimental channels. A needle valve (Swagelok, Solon, OH, United States) regulated flow rate in the baseline channel and a mass flow controller (MC50 SLP, Alicat Scientific Inc., Tucson, AZ, United States), calibrated using a soap-bubble flow meter (Giliblator-2; Sensidyne, St. Petersburg, FL, United States), regulated flow rates in the experimental channel. To maximise air mixing, and minimise any potential convective cooling at higher flow rates, we positioned the air inlet near

the top of the metabolic chamber with an elbow joint facing upward and the air outlet below the mesh platform. Flow rates ($13\text{--}65\text{ L min}^{-1}$) were adjusted to maintain humidity at a dewpoint $<-5^\circ\text{C}$ within the chamber, depending on T_{air} , M_b and individual behaviour, while still allowing for the accurate measurements of differences in water vapour and CO_2 between incurrent and excurrent air. A respirometry multiplexer (model MUX3-110118M, Sable Systems) in manual mode and an SS-3 Sub-sampler (Sable Systems) subsampled excurrent air from the baseline and chamber air. Subsampled air was pulled through a $\text{CO}_2/\text{H}_2\text{O}$ analyser (model LI-840A; LI-COR, Lincoln, NE, United States). The $\text{CO}_2/\text{H}_2\text{O}$ analyser was regularly zeroed using nitrogen, and spanned for CO_2 using a certified calibration gas (CO_2 concentration = 2,000 ppm; Afrox, South Africa). We regularly zeroed the H_2O sensor of the LI-840A using nitrogen and spanned it using air with a known dewpoint produced by a dewpoint generator (DG-4; Sable Systems). The system's tubing was Bev-A-Line IV tubing (Thermoplastic Processes Inc.). An analogue-digital converter (model UI-3; Sable Systems) digitised voltage outputs from the thermistor probes and analysers, which were recorded every 5 s using Expedata software (Sable Systems).

Experimental Protocol

Following Czenze et al. (2020) we placed individuals in chambers prior to measurements to habituate. Before measurements commenced, individuals were held without food for at least 1 h in the metabolic chamber, and together with the preceding period of fasted captivity ensured birds were likely postabsorptive when data collection started (Karasov, 1990).

Measurements took place during the day and individuals were exposed to a stepped series of progressively higher T_{air} values over which $\dot{V}_{\text{CO}_2}$ and EWL were recorded. We started measurements with a baseline air subsample until water and CO_2 readings were stable (~5 min). Chamber excurrent air was then subsampled once T_{air} had stabilised at the target value. We recorded data at this T_{air} until $\dot{V}_{\text{CO}_2}$ and H_2O traces were stable for at least 10 min, with individuals spending approximately 20–25 min at the target T_{air} before we switched back to baseline air for another 5 min. Trials began at 28°C with 4°C increases until 40°C and then 2°C increases until a maximum of 60°C (the maximum T_{air} our thermistors could accurately measure). We monitored individuals continuously using an infrared video camera. Individuals were removed from the chamber when they reached their thermal endpoints [i.e., loss of coordination or balance, sudden and rapid decrease in EWL or resting metabolic

rate (RMR), rapid and uncontrolled increase in T_b to values $>45^\circ\text{C}$] or displayed sustained escape behaviours like agitated jumping. In the case of the sandgrouse, measurements were terminated at T_{air} slightly above 60°C . Once an individual was removed from the chamber we quickly dabbed its underbelly with ethanol to facilitate rapid cooling and held it under chilled air produced by an air conditioner. Once the individual's T_b was stable ($40\text{--}42^\circ\text{C}$), we offered it water using a syringe, and placed it back in its cage to rest, with *ad libitum* water available. The bird was later released at the site of capture. This experimental protocol has been used previously on birds and bats and individuals recaptured several days or weeks post-release showed no adverse effects (Kemp and McKechnie, 2019; Czenze et al., in press).

Data Analysis

We used the R package *segmented* (Muggeo, 2008) to perform a broken stick regression analysis in R 3.5.2 (R Core Team, 2018) to identify inflection points for EWL, RMR, T_b , and EHL/MHP. To determine whether a broken-stick regression fit the data better than a simple linear model, we compared broken-stick models against generalised mixed-effect models created in R package *nlme* (Pinheiro et al., 2009) using ANOVA. If we retained a broken stick regression and significant inflections occurred, we analysed data below and above inflection points separately when

estimating slopes for the relationships of EWL, RMR, EHL/MHP, and T_b as functions of T_{air} . We created general linear mixed-effect models for each species using the R package *nlme* to test for an effect of T_{air} on each response variable above inflection points. We accounted for the repeated measures design of our study (i.e., measurements at multiple T_{air} values per individual) by including individual identity as a random factor in all analyses. Initial models contained T_{air} , M_b , and the $T_{\text{air}}:M_b$ interaction and model selection was performed using the “dredge” function in the *MuMIn* package (Bartoń, 2013). We selected the model with the highest rank among competing models using Akaike weights and Akaike information criterion values corrected for small sample size (AICc) (Burnham and Anderson, 2002). Body mass did not emerge as an important predictor for any of the response variables of any species. We assessed significance at $\alpha < 0.05$ and values are presented as mean $\pm$ SD.

RESULTS

Body and Air Temperature

Normothermic T_b at thermoneutrality varied by $\sim 1.4^\circ\text{C}$ among species, from 39.1 ± 0.4 ($^\circ\text{C}$) in eagle-owls to 40.5 ± 0.4 ($^\circ\text{C}$) in thick-knees (Figure 1 and Table 2). All three species showed significant increases in T_b above normothermic levels when

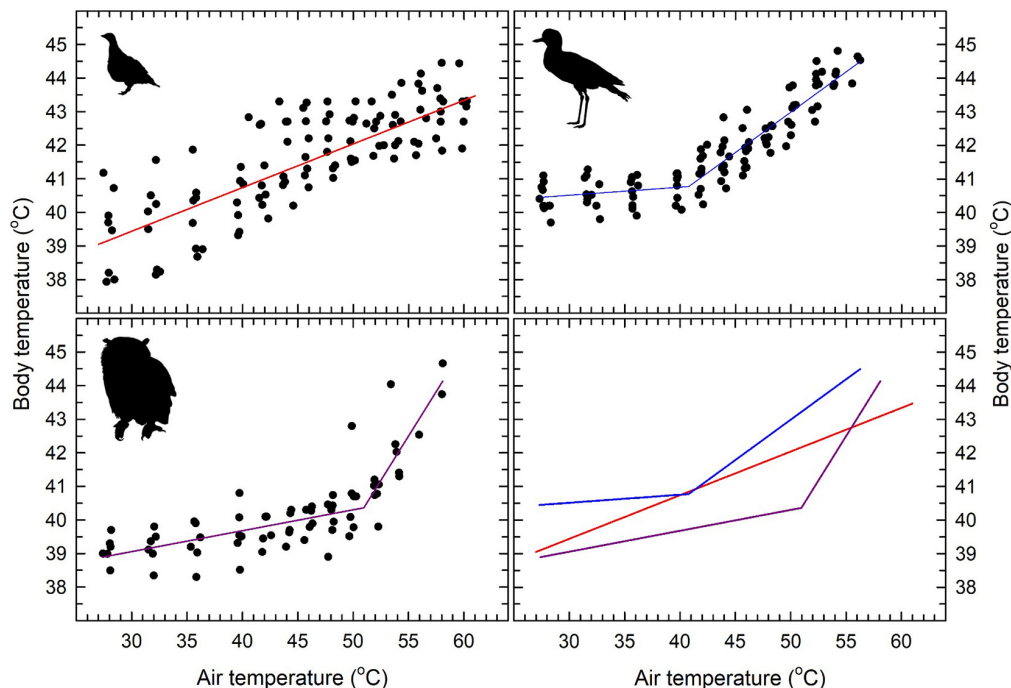


FIGURE 1 | Relationships between body temperature (T_b) and air temperature (T_{air}) in three species from the southern African arid zone: Namaqua sandgrouse (*Pterocles namaqua*; top left), spotted thick-knees (*Burhinus capensis*; top right) and spotted eagle-owls (*Bubo africanus*; lower left). The solid lines show a linear regression model (sandgrouse) or segmented linear regression model (thick-knees and owls). Inflection T_{air} values and slopes for relationships between T_b and T_{air} are provided in Table 2. The lower right panel shows the regression models for all three species for comparative purposes. The bird silhouettes were obtained from <http://phylopic.org>: *Pterocles* (Tarique Sani, John E. McCormack, Michael G. Harvey, Brant C. Faircloth, Nicholas G. Crawford, Travis C. Glenn, Robb T. Brumfield and T. Michael Keeseey), *Burhinus* (Auckland Museum) and *Bubo* (Lukasiniho). They are reproduced here under Creative Commons Licences (sandgrouse and thick-knee: <https://creativecommons.org/licenses/by/3.0/>; owl: <https://creativecommons.org/licenses/by-nc-sa/3.0/>).

TABLE 2 | Variables related to interactions between body temperature, metabolic heat production and evaporative heat dissipation as functions of air temperature in three species from the arid zone of southern Africa, quantified using flow-through respirometry: Namaqua sandgrouse (*Pterocles namaqua*), spotted thick-knees (*Burhinus capensis*) and spotted eagle-owls (*Bubo africanus*).

	Namaqua sandgrouse	Spotted thick-knee	Spotted eagle-owl
Body mass (g)	165.8 ± 17.2 (8)	434.3 ± 34.4 (10)	667.9 ± 77.1 (7)
Body temperature			
Min. T_b (°C)	39.4 ± 1.2 (8)	40.5 ± 0.4 (9)	39.1 ± 0.4 (6)
Inflection T_{air} (°C)	N/A	40.5 ± 0.05 (10)	51.1 ± 0.05 (7)
T_b versus T_{air} slope (°C)	0.13 ^a	0.24	0.53
Max. T_b (°C)	43.2 ± 0.8 (7)	44.3 ± 0.4 (3)	44.2 (2)
Max. T_{air} (°C)	60 (7)	56 (3)	58 (3) 60 (1)
T_b at onset of gular flutter (°C)	41.5 ± 1.4 (9)	41.9 ± 0.4 (9)	39.4 ± 0.6 (5)
T_{air} at onset of gular flutter (°C)	42.5 ± 4.7 (9)	44.5 ± 2.8 (9)	36.7 ± 4.1 (5)
95th percentile $T_b > T_{air}$ (°C)	42.6	41.2	40.8
Resting metabolic rate			
Min. RMR (W)	0.83 ± 0.14 (8)	2.03 ± 0.34 (3)	2.38 ± 0.55 (7)
T_{uc} (°C)	N/A	47.3 ± 0.01 (10)	49.2 ± 0.02 (7)
RMR slope (mW °C ⁻¹)	N/A	417.6	314.0
Max. RMR (W)	1.14 ± 0.18 (8)	5.61 ± 1.56 (3)	4.49 ± 1.99 (3) 5.16 (1)
Max. RMR/min. RMR	1.37	2.76	1.96
Evaporative water loss			
Min. EWL (g h ⁻¹)	1.01 ± 0.66 (8)	1.83 ± 0.57 (10)	1.79 ± 0.65 (7)
Inflection T_{air} (°C)	37.9 ± 1.70 (8)	42.7 ± 0.55	37.3 ± 1.55 (7)
EWL slope (g h ⁻¹ °C ⁻¹)	0.28	1.51	0.74
Max. EWL (g h ⁻¹)	7.48 ± 1.90 (7)	23.52 ± 11.35 (5)	14.04 (2)
Max. EWL/min. EWL	7.41	12.85	7.80
Min. EHL/MHP	0.66 ± 0.44 (8)	0.56 ± 0.17 (10)	0.52 ± 0.2 (7)
EHL/MHP inflection $T_{air} - T_b$ (°C)	-0.2 ± 0.04 (8)	-2.3 ± 1.54 (10)	-5.2 ± 1.25 (7)
EHL/MHP slope (°C ⁻¹)	0.22	0.20	0.14
Max. EHL/MHP	5.49 ± 1.74 (7)	3.15 ± 0.79 (9)	2.75 ± 0.8 (6)

T_b , body temperature; T_{air} , air temperature; RMR, resting metabolic rate; EWL, evaporative water loss; EHL, evaporative heat loss; MHP, metabolic heat production; Max., maximum; Min., minimum. Values are presented as mean ± SD, with sample sizes in parentheses. Unless otherwise indicated, slopes are for relationships at T_{air} values above listed T_{air} inflection points. In some cases, single values are listed if they were substantially higher than averages.

^aNo inflection occurred and slope is for T_b over the entire range of T_{air} .

exposed to higher T_{air} (Figure 1). In thick-knees and eagle-owls, significant inflections above which T_b increased rapidly occurred at $T_{air} = 40.7^\circ\text{C}$ and $T_{air} = 50.9^\circ\text{C}$, respectively (Table 2 and Figure 1). At T_{air} values above these inflection points, T_b increased significantly ($t = 16.94$, $P < 0.001$ and $t = 7.36$, $P < 0.001$, respectively) and in a linear fashion in thick-knees and eagle-owls. In sandgrouse, on the other hand, no inflection was evident and a linear model provided the best fit for the relationship between T_b and $T_{air} \geq 32.5^\circ\text{C}$ [the value we assumed to represent this species' lower critical limit of thermoneutrality (Figure 2)]. Over this T_{air} range, T_b increased significantly ($t = 18.53$, $P < 0.001$) to a maximum of $43.2 \pm 0.8^\circ\text{C}$ ($n = 7$). Mean maximum T_b values at very high T_{air} varied among the three species from 43.2°C to 44.3°C (Table 2). Heat tolerance limits (HTL, i.e., maximum T_{air} tolerated before thermal endpoints reached) varied from 56°C in thick-knees to 60°C in eagle-owls and Namaqua sandgrouse (Table 2), although HTL for the latter species may be even higher in some individuals.

Resting Metabolic Rate

Minimum thermoneutral RMR values varied from 0.83 W in Namaqua sandgrouse to 2.38 W in the eagle-owl (Table 2). Namaqua sandgrouse did not show a significant inflection in

RMR, which did not vary significantly with T_{air} (Figure 2). In contrast, significant inflections in RMR were evident for both thick-knees and eagle owls (Table 2), above which linear increases in RMR were significant ($t = 9.15$, $P < 0.001$ and $t = 4.1$, $P < 0.001$, respectively; Figure 2). These inflection T_{air} values, which correspond to the upper critical limits of thermoneutrality (T_{uc}), were $T_{air} = 47.3^\circ\text{C}$ in thick-knees and $T_{air} = 49.2^\circ\text{C}$ in eagle-owls. Maximum RMR at the highest T_{air} achieved was equivalent to 137%, 196%, and 276% of minimum thermoneutral values in Namaqua sandgrouse, eagle-owls and thick-knees, respectively (Figure 2). Mass-specific maximum RMR in thick-knees was ~85% higher compared to sandgrouse or eagle-owls (Table 3).

Evaporative Water Loss

Gular flutter commenced at T_{air} between 36.7°C and 44.5°C and T_b between 39.4°C and 42.4°C (Table 2). Minimum EWL varied nearly 2-fold from 1.01 g h^{-1} in Namaqua sandgrouse to 1.83 g h^{-1} in thick-knees (Table 2 and Figure 3). All species showed clear inflection points above which EWL increased rapidly and linearly; these varied from $T_{air} = 33.9^\circ\text{C}$ in Namaqua sandgrouse to $T_{air} = 42.8^\circ\text{C}$ in thick-knees (Table 1 and Figure 3). Increases in EWL were significant for sandgrouse ($t = 19.94$, $P < 0.001$),

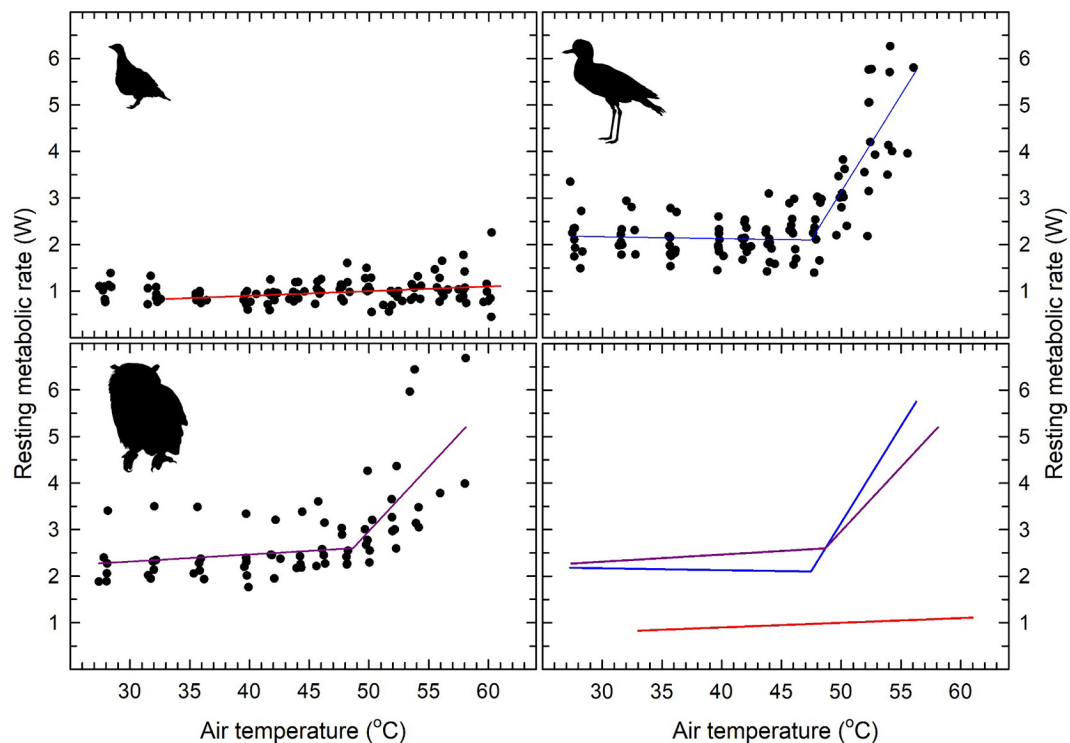


FIGURE 2 | Resting metabolic rate (RMR) showed a clear inflection (i.e., upper critical limit of thermoneutrality; T_{uc}) in spotted thick-knees (*Burhinus capensis*; top right) and spotted eagle-owls (*Bubo africanus*; lower left), but no inflection was evident for Namaqua sandgrouse (*Pterocles namaqua*; top left). Segmented linear regression models are shown for thick-knees and owls using solid lines, whereas the relationship between T_{air} and RMR in sandgrouse was non-significant. Inflection T_{air} values and slopes for relationships between T_b and T_{air} are provided in **Table 2**. The lower right panel shows patterns for all three species for comparative purposes. Sources of the bird silhouettes are provided in the **Figure 1** legend.

TABLE 3 | Mass-specific resting metabolic rate (RMR) and evaporative water loss (EWL) at high air temperature in three species from the arid zone of southern African: Namaqua sandgrouse (*Pterocles namaqua*), spotted thick-knees (*Burhinus capensis*) and spotted eagle-owls (*Bubo africanus*).

	Namaqua sandgrouse	Spotted thick-knee	Spotted eagle-owl
RMR			
Min. RMR ($mW g^{-1}$)	5.06 ± 0.78 (8)	4.69 ± 0.55 (9)	3.57 ± 0.74 (7)
RMR slope ($mW g^{-1}^{\circ}C^{-1}$)	N/A	0.93	0.58
Max. RMR ($mW g^{-1}$)	6.96 ± 1.84 (8)	12.16 ± 3.1 (3)	7.07 ± 3.53 (3)
EWL			
Min. EWL ($mg h^{-1} g^{-1}$)	6.5 ± 5.3 (8)	4.2 ± 1.2 (10)	2.7 ± 1.12 (7)
EWL slope ($mg h^{-1} g^{-1}^{\circ}C^{-1}$)	1.8	3.4	1.39
Max. EWL ($mg h^{-1} g^{-1}$)	45.2 ± 10.8 (7)	51.0 ± 24.2 (3)	26.05 ± 13.5 (6)

RMR, resting metabolic rate, EWL, evaporative water loss, EHL, evaporative heat loss, MHP, metabolic heat production, Max., maximum, Min., minimum. Values are presented as mean $\pm$ SD, with sample sizes in parentheses.

thick knees ($t = 13.67$, $P < 0.001$), and eagle owls ($t = 19.59$, $P < 0.01$). Maximum rates of EWL ranged from $7.48 g h^{-1}$ in Namaqua sandgrouse to $23.52 g h^{-1}$ in thick-knees, with the highest mass-specific values in thick-knees (**Table 3**). The fractional increase in EWL (i.e., the ratio of maximum EWL to minimum EWL) ranged from ~ 7.41 for Namaqua sandgrouse to ~ 12.85 for thick-knees (**Table 2**).

The relationships between evaporative heat loss (EHL)/metabolic heat production (MHP) and $T_{air}-T_b$ gradients (**Figure 4**), were characterised by significant inflections for all

species (**Table 2**). Above these inflection points, EHL/MHP increased linearly with increasing $T_{air}-T_b$, with relationships significant for sandgrouse ($t = 15.63$, $P < 0.001$), thick knees ($t = 11.62$, $P < 0.001$) and eagle owls ($t = 11.30$, $P < 0.001$). Maximum evaporative cooling efficiency (i.e., maximum EHL/MHP) ranged from 2.75 for eagle-owls to 5.49 in Namaqua sandgrouse (**Figure 4**). However, these EHL/MHP values were calculated assuming RER = 0.71 (i.e., lipid catabolism), and could be higher if individuals used carbohydrates (i.e., RER = 1.00). In this case, maximum EHL for eagle-owls and

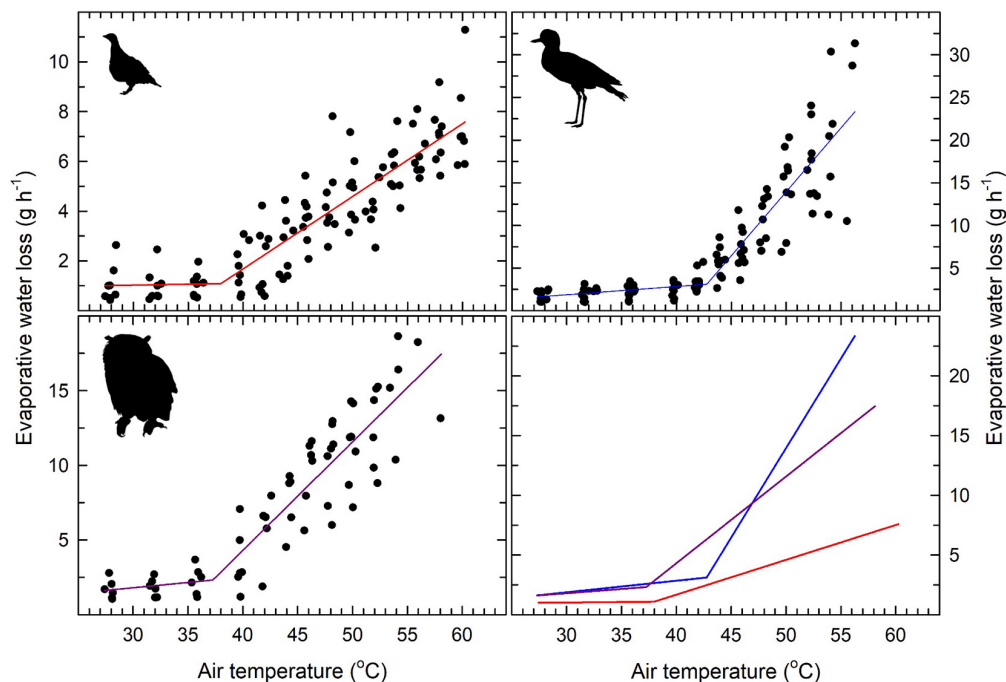


FIGURE 3 | In three medium- to large-sized species from the southern African arid zone, Namaqua sandgrouse (*Pterocles namaqua*; top left), spotted thick-knees (*Burhinus capensis*; top right) and spotted eagle-owls (*Bubo africanus*; lower left), relationships between air temperature (T_{air}) and evaporative water loss (EWL) were characterised by significant inflections between $T_{\text{air}} = 37\text{--}43^{\circ}\text{C}$. Above these inflections, EWL increased linearly to maximum values equivalent to 7.4, 12.9 and 7.8 X minimum thermoneutral values in Namaqua sandgrouse, thick-knees and eagle-owls, respectively. The solid lines show segmented linear regression models fitted to each data set, with inflection T_{air} values and slopes for relationships between EWL and T_{air} provided in **Table 2**. The lower right panel shows the regression models for all three species plotted on the same axes for comparative purposes. Sources of the bird silhouettes are provided in the **Figure 1** legend.

Namaqua sandgrouse would shift upward to 3.66 and 7.30, respectively. Visual inspection of the data in **Figure 4**, however, does not support this possibility. Theoretically, evaporative heat loss and metabolic heat production should be equal (i.e., $\text{EHL}/\text{MHP} = 1.0$) when $T_{\text{air}} = T_b$. Currently $\text{EHL}/\text{MHP} = 1.0$ occurs at approximate $T_{\text{air}} - T_b$ values of -1.5 , -2.0 , and -4.5°C for thick-knees, eagle-owls, and Namaqua sandgrouse, respectively. Recalculating EHL/MHP using $\text{RER} > 0.71$ would shift these values even further below $T_{\text{air}} - T_b = 0$, suggesting our assumption that $\text{RER} = 0.71$ is likely correct.

DISCUSSION

In the three species we examined here, rapid increases in EWL facilitated the defence of T_b below lethal limits even at $T_{\text{air}} > 55^{\circ}\text{C}$. Although these experimental T_{air} values are well above the range our study species ever experience naturally, our data provide insights into the avoidance of lethal hyperthermia at midday operative temperatures in unshaded microsites, where intense solar radiation may result in microclimates substantially hotter than T_{air} alone (Bakken, 1976; Robinson et al., 1976). Together with maximum EHL/MHP values of 2.8–5.5, the high heat tolerance limits of these species support the notion that gular flutter provides the basis for pronounced evaporative cooling and heat tolerance, although quantifying the contribution of gular

flutter was precluded here by us not partitioning respiratory and cutaneous EWL. We found less support for the idea that patterns of thermoregulation at high T_{air} remain similar within genera and families, with patterns of thermoregulation in *P. namaqua* and *B. africanus* differing to varying extents from those of previously-studied sandgrouse and owls, respectively. We discuss these differences in more detail below.

Patterns of Thermoregulation

Relationships between T_b and T_{air} conformed to typical avian patterns in thick-knees and eagle-owls, although the inflection T_{air} for eagle-owls is well above the range of $30\text{--}40^{\circ}\text{C}$ typical of most species investigated to date (McKechie et al., 2017; McWhorter et al., 2018; Smit et al., 2018; Czenze et al., 2020). The eagle-owl inflection is also substantially higher than those reported for two smaller owls; $T_{\text{air}} = 37.3^{\circ}\text{C}$ in 101-g western screech-owls (*Megascops kennicottii*) and $T_{\text{air}} < 30^{\circ}\text{C}$ in 40-g elf owls (*Micrathene whitneyi*; Talbot et al., 2018). In contrast to the thick-knees and eagle-owls, no significant inflection occurred in the relationship between T_b and T_{air} in Namaqua sandgrouse, a pattern qualitatively similar to those reported for *P. namaqua* and double-banded sandgrouse (*P. bicinctus*) held in outdoor enclosures during summer in the Namib Desert (Thomas and Maclean, 1981), as well as black-bellied sandgrouse (*P. orientalis*) at T_{air} between 10 and 45°C (Hinsley et al., 1993).

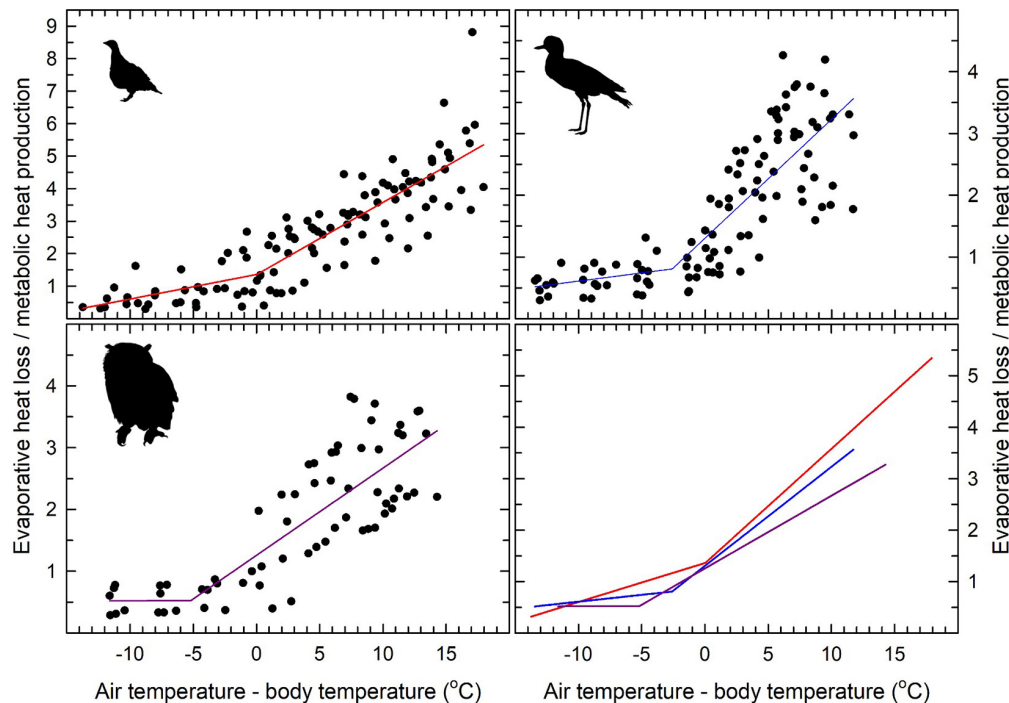


FIGURE 4 | Ratio of evaporative heat loss (EHL) and metabolic heat production (MHP) as a function of the gradient between air temperature (T_{air}) and body temperature (T_b) in Namaqua sandgrouse (*Pterocles namaqua*; top left), spotted thick-knees (*Burhinus capensis*; top right) and spotted eagle-owls (*Bubo africanus*; lower left). Segmented linear regression models fitted to each data set are shown using solid lines, with inflection T_{air} values and slopes for relationships between EHL/MHP and $T_{\text{air}} - T_b$ provided in **Table 2**. The lower right panel shows the regression models for all three species plotted on the same axes for comparative purposes. Sources of the bird silhouettes are provided in the **Figure 1** legend.

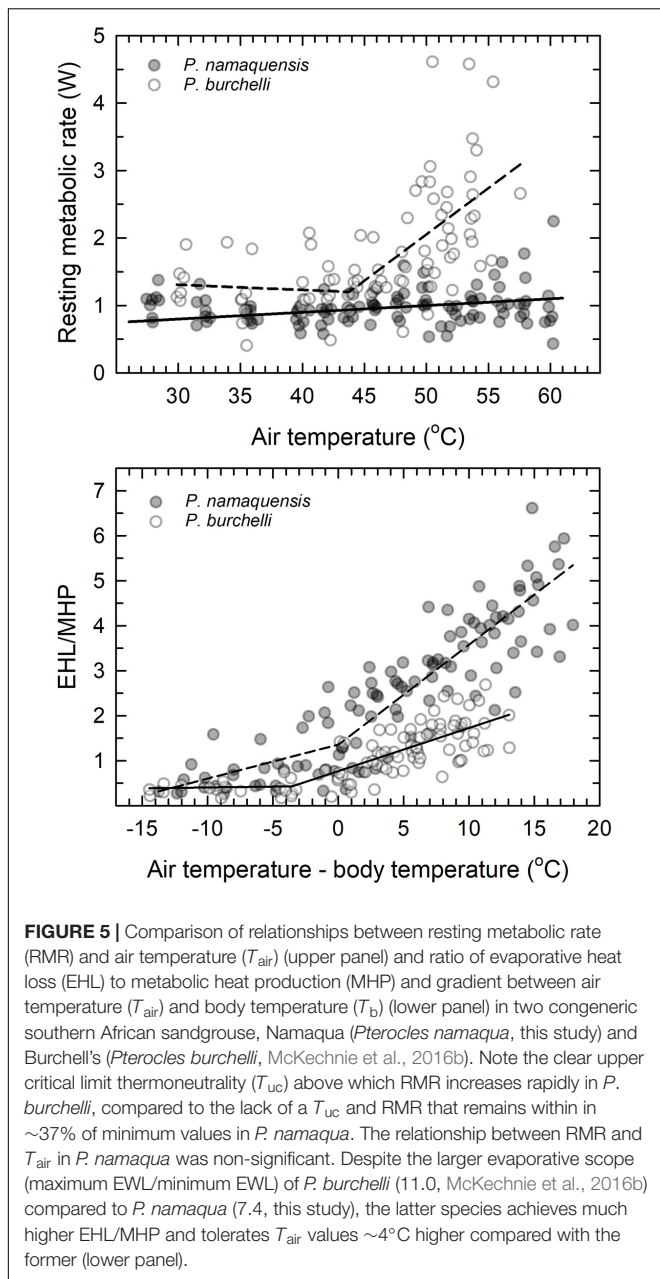
Maximum T_b at thermal endpoints is within the range documented for non-passerines (McKechnie et al., 2021a), with values $>44^\circ\text{C}$ for thick-knees and eagle-owls near the upper end of this range. The heat tolerance limits of all three species are higher than those of most non-passerines and all passerines, with the values of 58°C and 60°C in eagle-owls and Namaqua sandgrouse, respectively, comparable to those of some caprimulgids and columbids, the avian taxa with the highest documented heat tolerances (Marder and Arieli, 1988; O'Connor et al., 2017; Talbot et al., 2017).

The linear increases of EWL with $T_{\text{air}} > T_b$ also followed patterns typical of birds, although evaporative scope (i.e., maximum EWL/minimum EWL) was nearly twice as high in thick-knees (12.9) compared to Namaqua sandgrouse (7.4) or eagle-owls (7.8). Moreover, maximum rates of EWL for thick-knees (23.5 g h^{-1} at $T_{\text{air}} = 56^\circ\text{C}$) exceed by a substantial margin the highest rates of EWL reported for the much larger (~ 3 -fold) MacQueen's bustard (Tieleman et al., 2002). Evaporative water loss rates of thick-knees were equivalent to $5.4\% M_b \text{ h}^{-1}$, higher-than-expected fractional values more similar to those typically seen in small passerines (Wolf and Walsberg, 1996; McKechnie et al., 2017; Czenze et al., 2020).

Increases in RMR associated with evaporative heat dissipation at high T_{air} varied substantially. In Namaqua sandgrouse, maximum RMR at $T_{\text{air}} = 60^\circ\text{C}$ was only 37% higher than minimum thermoneutral values, a pattern similar to those

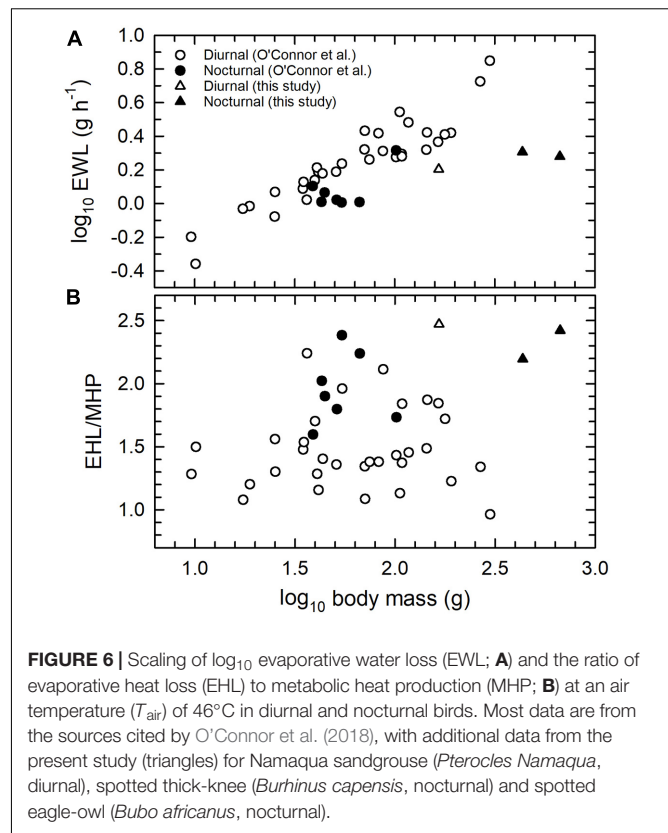
observed in some caprimulgids (O'Connor et al., 2017), columbids (McKechnie et al., 2016a,b) and at $T_{\text{air}} \leq 55^\circ\text{C}$ in MacQueen's bustard (Tieleman et al., 2002). Among other sandgrouse species, no daytime T_{uc} was evident in *P. orientalis* nor *P. bicinctus* at T_{air} values below $\sim 43^\circ\text{C}$ (Hinsley, 1992; Hinsley et al., 1993), suggesting measurements may not have reached T_{air} high enough to elicit a T_{uc} in either of these species. The very high T_{uc} of eagle-owls ($T_{\text{air}} = 49^\circ\text{C}$) is similar to the $T_{\text{uc}} = 46.4^\circ\text{C}$ reported for western screech owls (Talbot et al., 2018) and other taxa, including lilac-breasted roller (*Coracias caudatus*: $T_{\text{uc}} = 47.5^\circ\text{C}$; Smit et al., 2018) and several caprimulgids and columbids (Smith et al., 2015; McKechnie et al., 2016a; Talbot et al., 2017).

With the exception of maximum EHL/MHP = 3.4 in lilac-breasted rollers, the values we report here are the highest yet documented among birds other than caprimulgids and columbids (reviewed by McKechnie et al., 2021a). In particular, maximum EHL/MHP = 5.5 in Namaqua sandgrouse is the highest avian value measured to date, exceeding values of 4.7 and 5.2 previously reported for Namaqua doves (*Oena capensis*; McKechnie et al., 2016a) and Rufous-cheeked nightjars (*Caprimulgus rufigena*; O'Connor et al., 2017), respectively. We are not aware of published data on maximum EHL/MHP in Charadriiformes, but our value of 3.2 for thick-knees raises the possibility that the capacity for evaporative cooling may also be pronounced in this order (e.g., Grant, 1982).



Interspecific Variation Between Sandgrouse

The data currently available suggest patterns of thermoregulation in the heat are phylogenetically conserved within avian orders. The large differences in patterns of thermoregulation at very high T_{air} between *P. namaqua* and the congeneric Burchell's sandgrouse (*P. burchelli*, McKechnie et al., 2016b) were, therefore, surprising. Whereas RMR increased sharply to values equivalent to $\sim 250\%$ of minimum thermoneutral levels in *P. burchelli*, the RMR of *P. namaqua* remained within 37% of minimal levels (Figure 5). Consequently, maximum EHL/MHP was substantially higher in *P. namaqua* (5.5 at $T_{air} = 60^\circ\text{C}$) compared to *P. burchelli* (2.0–2.7 at $T_{air} = 56^\circ\text{C}$). The



rapid increases in RMR and modest maximum EHL/MHP of *P. burchelli* bear a strong resemblance to the pattern typical of passerines, which lack gular flutter or rapid cutaneous water loss and rely on panting for evaporative heat dissipation. In contrast, the corresponding patterns for *P. namaqua* resemble the extremely efficient cooling characteristic of columbids (Smith et al., 2015; McKechnie et al., 2016a) and caprimulgids (O'Connor et al., 2017; Talbot et al., 2017). The sandgrouse provide the first indication that fundamental differences in evaporative cooling processes can occur within an order, or even a single genus.

We speculate the differences between these two species reflect the more arid distribution of *P. namaqua*, which likely experiences stronger selection to minimise energy and water requirements. The distribution of *P. burchelli* is centred on the Kalahari basin, spanning arid savanna in the south to mopane (*Colophospermum mopane*) woodlands in the north. Much of the distribution of *P. namaqua*, on the other hand, is more arid, including the Nama Karoo and hyperarid Namib Desert. With mean annual precipitation of $\sim 100 \text{ mm y}^{-1}$, our present study site is considerably more arid than any part of the range of *P. burchelli* (Lloyd, 2005). The methods used to quantify thermoregulatory responses in both species were identical, but we cannot rule out possibilities such as a greater stress response in *P. burchelli* compared to *P. namaqua*. However, in this scenario we would expect higher T_b under thermoneutral conditions in *P. burchelli* on account of stress-induced hyperthermia

TABLE 4 | Comparison of body temperature (T_b), evaporative water loss (EWL), resting metabolic rate (RMR) and ratio of evaporative heat loss (EHL) to metabolic heat production (MHP) at air temperatures (T_{air}) of approximately 50°C in six species with body mass (M_b) > 250 g: MacQueen's bustard (*Chlamydotis macqueenii*, $n = 7$), spotted eagle-owl (*Bubo africanus*), brown-necked raven (*Corvus ruficollis*, $n = 11-16$), chukar (*Alectoris chukar*), spotted thick-knee (*Burhinus capensis*) and galah (*Eolophus roseicapilla*, $n = 7$).

Species	M_b g	T_b °C	EWL g h ⁻¹	RMR W	EHL/MHP	Source
MacQueen's Bustard	1245	41.5	8.64	3.26	1.77	Tieleman et al. (2002)
Spotted Eagle-Owl	668	40.6	11.29	2.96	2.61	Present study
Brown-necked Raven	610	44.7	17.84	7.19	1.67	Marder (1973)
Chukar	475	43.4	7.35	2.73	1.82	Marder and Bernstein (1983)
Spotted Thick-knee	434	42.9	14.35	3.05	3.02	Present study
Galah	266	42.3	7.19	2.96	1.62	McWhorter et al. (2018)

In the case of *C. ruficollis*, data were collected at $T_{air} = 48^\circ\text{C}$.

(Cabanac and Briese, 1992; Cabanac and Aizawa, 2000; Nord and Folkow, 2019). The similarity of both normothermic T_b (*P. burchelli*: 39.0°C and *P. namaqua*: 39.4°C) and maximum T_b (*P. burchelli*: 43.6°C and *P. namaqua*: 43.2°C) argues against the notion that the divergent responses of these congeners reflect an artefact of stress responses to the experimental conditions.

Functional Links With Nocturnality in Thick-Knees and Owls

The high daytime environmental temperatures nocturnal birds endure in hot habitats, combined with their lack of drinking during the day, led O'Connor et al. (2018) to hypothesise that nocturnal species have evolved more economical evaporative cooling and reduced EWL to minimise dehydration risk during the day. An analysis of data for 32 diurnal and 7 nocturnal species (two owls, four caprimulgids and one owl-nightjar) provided weak support: slopes of EWL between $T_{air} = 40$ and 46°C were significantly lower in nocturnal species, but neither log₁₀ EWL nor EHL/MHP at $T_{air} = 46^\circ\text{C}$ differed significantly with activity period (O'Connor et al., 2018). Our data for thick-knees and eagle-owls generally support O'Connor et al.'s (2018) hypothesised link between nocturnality and more economical evaporative cooling, and specifically the predictions of lower EWL (Figure 6A) and higher EHL/MHP (Figure 6B) in nocturnal species, as well as these authors' contention that their results reflected a small sample size for nocturnal species.

Thermoregulation in the Heat: Interspecific Variation

The data we present here reveal that representatives of three non-passerine orders can achieve rapid evaporative heat dissipation and tolerance of $T_{air} = 55-60^\circ\text{C}$, similar to the performance of smaller species in highly heat tolerant taxa such as Caprimulgiformes and Columbiformes. Most species in which heat tolerance has been investigated so far are smaller than 100 g, and interspecific variation in interactions between evaporative heat loss and metabolic heat production at $T_{air} > 45^\circ\text{C}$ in larger species has received little attention (Table 4). The contrast between MacQueen's bustards (Tieleman et al., 2002) and brown-necked ravens (Marder, 1973) is particularly striking (Table 4). Despite being twice as large, bustards' EWL and RMR were equivalent to <50% of corresponding values in ravens,

whereas EHL/MHP was similar and T_b was 3.2°C higher in ravens (Table 4). Although the relative humidity experienced by ravens (<30%, Marder, 1973) may have been higher than for bustards (<22%, Tieleman et al., 2002), we suspect these differences primarily reflect the ravens' reliance on panting and the constraints imposed by this relatively inefficient avenue of evaporative cooling (McKechnie et al., 2021a). Spotted eagle-owls (present study) maintained the lowest T_b at high T_{air} of any of the six species, via a combination of intermediate rates of EWL and low RMR (Table 4).

The pronounced interspecific variation in EWL at $T_{air} = 50^\circ\text{C}$ reveals that vulnerability to lethal dehydration during extreme heat events differs considerably among the six species listed in Table 4. Assuming birds can tolerate losing water equivalent to 15% of M_b [following Albright et al. (2017) and others], survival times at $T_{air} = 50^\circ\text{C}$ vary from 4.53 h (spotted thick-knee) and 5.13 h (brown-necked raven) to 8.88 h (spotted eagle-owl) and 21.61 h (MacQueen's bustard). That estimated survival times for these species are >4 h at this extremely high T_{air} emphasizes the effect of M_b on the risk of lethal dehydration during extreme heat events; larger birds are at considerably lower risk of mortality via dehydration tolerance limits being exceeded (McKechnie and Wolf, 2010).

CONCLUSION

Our investigation of heat tolerance in three phylogenetically diverse birds with M_b of 170–680 g reveals pronounced evaporative cooling capacities and tolerance of extremely high T_{air} during acute heat exposure. These data add to evidence that many non-passerine taxa possess substantially more efficient evaporative cooling and greater heat tolerance compared to passerines (reviewed by McKechnie et al., 2021a). Pronounced heat tolerance in many non-passerines, including the three species in the present study, appears to be functionally linked to the capacity for gular flutter, or in the case of the columbids, rapid cutaneous evaporative cooling. The loss of gular flutter during avian evolution left passerines and several other taxa with much more limited evaporative cooling capacity, which is thought to have contributed to the evolution of passerines' pronounced use of facultative hyperthermia (Gerson et al., 2019).

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 Animal Ethics Committee, University of Pretoria (protocol UP: EC057-18) and the Research Ethics and Scientific Committee of the South African National Biodiversity Institute (P18/37).

AUTHOR CONTRIBUTIONS

RK, ZC, BW, MF, and AM designed the study. ZC, MF, RK, and BvJ collected and analysed the data. ZC, MF, and AM led the

writing of the manuscript. All authors contributed to the article and approved the submitted version.

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How to Stay Cool: Early Acoustic and Thermal Experience Alters Individual Behavioural Thermoregulation in the Heat

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Climate change is pushing organisms closer to their physiological limits. Animals can reduce heat exposure – and the associated risks of lethal hyperthermia and dehydration – by retreating into thermal refuges. Refuge use nonetheless reduces foraging and reproductive activities, and thereby potentially fitness. Behavioural responses to heat thus define the selection pressures to which individuals are exposed. However, whether and why such behavioural responses vary between individuals remains largely unknown. Here, we tested whether early-life experience generates inter-individual differences in behavioural responses to heat at adulthood. In the arid-adapted zebra finch, parents incubating at high temperatures emit “heat-calls,” which adaptively alter offspring growth. We experimentally manipulated individual early life acoustic and thermal experience. At adulthood, across two summers, we then repeatedly recorded individual panting behaviour, microsite use, activity ($N = 2,402$ observations for 184 birds), and (for a small subset, $N = 23$ birds) body temperature, over a gradient of air temperatures (26–38°C), in outdoor aviaries. We found consistent inter-individual variation in behavioural thermoregulation, and show for the first time in endotherms that early-life experience contributes to such variation. Birds exposed prenatally to heat-calls started panting at lower temperatures than controls but panted less at high temperatures. It is possible that this corresponds to a heat-regulation strategy to improve water saving at high temperature extremes, and/or, allow maintaining high activity levels, since heat-call birds were also more active across the temperature gradient. In addition, microsite use varied with the interaction between early acoustic and thermal experiences, control-call birds from cooler nests using the cooler microsite more than their hot-nest counterparts, whereas the opposite pattern was observed in heat-call birds. Overall, our study demonstrates that a prenatal acoustic signal of heat alters how individuals adjust behaviourally to thermal challenges at adulthood. This suggests that there is scope for selection pressures to act differently across individuals, and potentially strengthen the long-term fitness impact of early-life effects.

Keywords: behavioural flexibility, reaction norm, developmental plasticity, trade-off, heat dissipation, heat tolerance

INTRODUCTION

Global temperatures are increasing at an unprecedented rate, and heatwaves are becoming more frequent and longer (IPCC, 2014). Climate change is transforming ecosystems across the globe and pushing organisms beyond their physiological limits (Conradie et al., 2019, 2020; McKechnie and Wolf, 2019; Stillman, 2019), fuelling population declines across taxa (Dirzo et al., 2014; Urban, 2015; Riddell et al., 2019; Rosenberg et al., 2019). Among endotherms, birds may be particularly at risk, due to their relatively small size and diurnal activity. In the short-term, extreme heat exposes organisms to an immediate risk of lethal dehydration and hyperthermia (McKechnie and Wolf, 2010; Albright et al., 2017), which has caused mass-mortality events, including in birds and bats (Welbergen et al., 2008; McKechnie et al., 2012, 2021). In the longer-term, high temperatures can impose important fitness costs through effects on reproduction and body condition (Cunningham et al., 2021; Oswald et al., 2021). Such selection pressures from high temperatures are exacerbated under climate change.

Yet, at any one place in time, selection by high temperatures does not operate equally across individuals. This is because individuals respond behaviourally to thermal challenges (Smit et al., 2016; Xie et al., 2017; Thompson et al., 2018; Pattinson et al., 2020). To avoid lethal hyperthermia, birds rely on thermoregulatory behaviours, such as panting, to dissipate heat. Panting allows dissipating excess heat through respiratory evaporative water loss (EWL) (Calder and King, 1974; Wolf and Walsberg, 1996; McKechnie and Wolf, 2019). Panting must thus be traded-off against water loss, and lethal dehydration risk. Importantly, however, organisms can considerably buffer their exposure to heat, by seeking thermal refuge into cooler microsites (Cunningham et al., 2015; Martin et al., 2015; Oswald et al., 2019). For instance, in rufous-eared warblers (*Malcorus pectoralis*), the environmental temperature in shaded off-ground microsites was up to 20°C cooler than sun-exposed sites on the ground, and individuals increased the time spent in these shaded microsites by 131% on hot days (Pattinson and Smit, 2017). However, microsite use physically constrains activity and foraging (Tieleman and Williams, 2002; Cunningham et al., 2015), with potential carry-over effects on body condition (du Plessis et al., 2012; van de Ven et al., 2019; Oswald et al., 2021) and breeding success (Nilsson and Nord, 2018; Sharpe et al., 2019; Cook et al., 2020; van de Ven et al., 2020). Behavioural responses to heat are therefore essential, because they define the selection pressures individuals are exposed to, and the life-history traits affected by selection. Surprisingly however, whether behavioural thermoregulation varies between individuals and the sources of such variation remain largely unknown.

Studies on passerines have increasingly revealed intra-specific variation in thermoregulation and heat tolerance between seasons (Noakes et al., 2016a; Oswald et al., 2018b), years (Noakes and McKechnie, 2019) and populations (Smit et al., 2013; Noakes et al., 2016b), following acclimatisation patterns to different climatic conditions. For inter-individual variation, however, we are aware of only two studies on behavioural thermoregulation. The onset of panting behaviour in zebra finches (*Taeniopygia guttata*) exposed to a standard increase

in air temperature (T_{air}) in a metabolic chamber is repeatable within individuals, but varies considerably between individuals, with some individuals starting panting at 27°C, while others do not pant until air temperature reaches 40°C (Pessato et al., 2020). This suggests that individuals may consistently vary in their thermoregulation strategies. In addition, in African passerines, microsite use varies with social status (Cunningham et al., 2017): dominant individuals used shaded sites more than subordinates in the territorial fawn-coloured lark (*Mirafra africanoides*) and the loosely social red-eyed bulbul (*Pycnonotus nigricans*), but not in the colonial sociable weaver (*Philetairus socius*) (Cunningham et al., 2017).

Such inter-individual variation in behavioural thermoregulation at adulthood may arise from the long-lasting effects of developmental conditions on phenotypes (i.e., developmental programming) (West-Eberhard, 2003; Durant et al., 2013; Groothuis et al., 2019). Surprisingly this has, to date, only been tested in ectotherms, where incubation temperatures affect thermoregulatory behaviours later in life, such as time spent basking or temperature selection (i.e., microsite preference) (reviewed in Refsnider et al., 2019). In endotherms, and specifically in birds, it was recently suggested that developmental programming for high temperatures may occur through prenatal acoustic communication (Mariette and Buchanan, 2016), as prenatal sounds adaptively alter development in a range of vertebrate and invertebrate species (Mariette and Buchanan, 2016; Noguera and Velando, 2019; Mariette et al., 2021). Notably, in the arid-adapted zebra finch, embryonic exposure to “heat-calls,” emitted by parents incubating at high temperatures, affects nestling growth under high postnatal nest temperatures, with positive effects on reproductive fitness at adulthood (Mariette and Buchanan, 2016). In addition, such prenatal heat-call exposure shifted thermal preferences in males, which preferred breeding in hotter nest sites than males exposed to control calls (Mariette and Buchanan, 2016). While this suggests long-term behavioural changes, we do not know whether prenatal acoustic signals could also affect individual behavioural responses to heat, and thereby modulate the selective pressures operating on these two phenotypes.

Here, we investigated whether individuals differ in their behavioural responses to heat at adulthood, and whether early acoustic and thermal experience contributes to this variation. We tested these hypotheses in adult male and female zebra finches from two replicate experiments, where individuals were prenatally exposed to heat-calls or control-calls in artificial incubators, and then reared under different postnatal nest temperatures. In addition to this experimental cohort (C_{exp}), we used a small unmanipulated control cohort (C_{cont}), to verify that inter-individual variation at adulthood also occurs when developmental conditions are not experimentally altered. Then, across two successive summers, we repeatedly recorded individual panting behaviour, microsite use and activity levels, along a gradient of air temperatures (25.8–37.6°C) in outdoor aviaries. We also simultaneously recorded the body temperature (T_b) of a small subgroup, while behaving freely in the aviaries. We predicted that (1) behavioural responses to heat consistently vary between individuals in both cohorts, and (2) that this variation partly arises from early acoustic and thermal experience

(tested in C_{exp}). Specifically, we expected birds exposed to heat-calls and/or reared in hot nests to be better adapted to high temperatures and therefore to: i) pant less, ii) spend less time in cooler microsites, and iii) remain more active, particularly at high temperatures.

MATERIALS AND METHODS

Experimental Subjects

We carried out the experiment at Deakin University, Geelong, Australia, during austral summers 2017–2018 and 2018–2019. In total, 214 wild-derived zebra finch adults (i.e., born in captivity but 10 to 12th generation descendants of wild individuals from Northern Victoria), were observed, while behaving freely in outdoor aviaries.

For the control cohort, C_{cont} , developmental conditions were not experimentally manipulated; individuals ($N = 30$ birds) were incubated and raised by their parents breeding in nest boxes in outdoor aviaries. Birds in the experimental cohorts, C_{exp} , were obtained from two playback experiments: one original experiment conducted in 2014 (Mariette and Buchanan, 2016) ($N = 55$ birds), and a replicate experiment conducted in 2017 ($N = 129$ birds). For both replicates, eggs were collected on laying day and replaced with dummy eggs. Freshly collected eggs were incubated in a main incubator at 37.5°C and 60% humidity. After 9 days, they were transferred to one of the two experimental incubators for the last 4–5 days of incubation. In each incubator, two speakers (Sennheiser HD439) externally connected to an amplifier (Digitech 18W) and an audio player (Zoom H4nSP) broadcast a playback of either contact calls (control) or heat-calls (treatment). Both call types are produced naturally by incubating parents, either when communicating with their partner or when experiencing heat, respectively. In addition, to allow normal stimulation of the auditory system, both playbacks also included whine calls, also produced by parents in the nest and characterised by a complex acoustic structure. The prenatal playbacks were played daily, from 9:30 to 18:30 (averaging 16 min of control- or heat- call per hour), until hatching. Eggs and sound cards were swapped daily between the two experimental incubators to prevent any incubator-specific effect. Further methodological details are provided in Mariette and Buchanan (2016).

Upon hatching, nestlings were identified (by clipping head down feathers) and returned to their parents or foster parents in nest boxes in outdoor aviaries. For the original experiment (Mariette and Buchanan, 2016), natural temperature variation caused by different sun exposure throughout the day resulted in a gradient of warm to hot nest-boxes [i.e., 3–6°C above air temperature during daytime (11:00–17:30)]. For the replicate experiment, nest temperatures were manipulated from hatching to 14 days post-hatch (dph) to obtain a larger gradient of temperatures (i.e., 1 to 8°C above daytime T_{air}). Specifically, we increased temperature in hot nests ($N = 68$) using a heat pad (Medi Heat Pack®) under the nest-box roof (when $T_{air} < 30^\circ\text{C}$) and a second (Hotteeze®) at the back of the nest (when $T_{air} < 25^\circ\text{C}$). We maintained some other nests cool ($N = 54$) using shading cloth, as well as a cool pad under the

roof (when $T_{air} > 25^\circ\text{C}$). For each brood, we calculated the average nest temperature above T_{air} (here shortened to “nest temperature differential,” T_{d-nest}), between 2 and 14 dph, as the average difference between the maximal daily nest temperature and the maximal daily air temperature [Bureau of Meteorology data, details in Section “Air Temperature (T_{air})”]. As in Mariette and Buchanan (2016), this nest temperature differential better represents the thermal microenvironment experienced during development, compared to raw nest temperatures.

Behavioural Observations

Each aviary included 3 top perches, at the front and back of the aviary, and a hanging feeder. The floor was made of concrete. Bird location was recorded as “perches” (i.e., perching on the perches or feeder) or “floor.” Both the perches and the floor below were in partial shade under a translucent fibreglass roof during the observations, but the floor was always at least 3.5°C cooler than the perches (see results). The perches and floor were therefore considered as two distinct microsites.

Individuals from C_{cont} were observed only during the first summer, and those from C_{exp} during both summers. Observations occurred on “hot days,” forecasted to exceed 26°C, corresponding to the minimum air temperature (i.e., minimum 29–32°C in the nest) at which heat-calls are produced by incubating parents in this population (Mariette and Buchanan, 2016). There were 10 days of observation in the first summer, between 18-Oct-2017 and 7-Feb-2018, and 9 days in the second summer, between 7-Dec-2018 and 2-Feb-2019. These observations were conducted during the hottest time of the day (between 11:00 and 17:00) when T_{air} exceeded 26°C (range: 25.8–37.6°C).

In total, we collected data during 40 “observation sessions” distributed across the 19 hot days, with 1 to 4 observation sessions per day. For each observation session, all aviaries were observed, in random order, for 10–20 min each. Within aviaries, we aimed to sight each individual once per session, in random order, to record their identity, behaviour, panting and location during 30 s of focal observation per bird. In total, we collected $N = 2,654$ individual observations (mean = 12.4 observations per individual), including $N = 1743$ and $N = 911$ individual observations in the first and second year respectively. Observations were made from 3 to 6 metres away, in a shaded corridor behind the aviary wire mesh, where birds are accustomed to human presence. Nonetheless, observations started after 15 minutes of habituation to the observer presence. Observers were blind to which treatment individual birds belonged.

For each individual observation, we recorded the bird identity, location, and behaviour (presence/absence) as follows: panting (bill open for > 5 s), perching (standing stationary on a perch), resting (crouching and/or closing eyes), hopping (short movements on or between close perches, or on the floor), flying (between front and back of the aviary, or to/from the floor), eating (pecking seeds in the feeder or on the floor), singing/calling (as indicated by sound, posture and throat movements). To obtain individual activity state (thereafter “activity”), behaviours were classified as either active (i.e., hopping, flying, eating or singing/calling) or inactive (perching or resting).

Predictor Variables for Temperature and Time During Observations

Air Temperature (T_{air})

Atmospheric air temperature (T_{air}) every 30 min was provided by the Australian Bureau of Meteorology (BOM), from the Breakwater Geelong Racecourse station, 6.7 km from the aviaries (station number 87184, latitude: -38.1737 , longitude: 144.3765 , elevation above sea level: 12.9 m).

Microsite Temperature (T_{loc})

We recorded the environmental temperature at the perches (T_{perch}) and 5 cm above the floor (T_{floor}) using temperature data loggers (Minnow-1.0TH, Senonics), in six of the ten aviaries (placed in alternate aviaries due to the limited number of loggers). This environmental temperature allows estimating the conditions experienced by the birds, with the integration of T_{air} with wind, solar and reflecting radiation (Cunningham et al., 2021). Temperature recordings (total $N = 5502$ at one-minute intervals) occurred on three sunny days (30-Oct-2018; 7 and 12-Dec-2018) between 11:00 and 17:00, with one thermometer on the floor and two on perches in each of three aviaries per day.

Across thermometer deployment days, the difference between T_{perch} and T_{air} (i.e., BOM data) increased linearly with air temperature. We therefore use the equation from this linear regression of T_{perch} over T_{air} to obtain the T_{perch} experienced by birds at any one time during observation days. Likewise, as the temperature differential between T_{perch} and T_{floor} decreased linearly with T_{air} , we used that linear regression to calculate the temperature differential during observations, and then subtracted it from T_{perch} (calculated above) to determine T_{floor} .

Body Temperature (T_b)

A subset of individuals ($N = 23$) from C_{exp} were equipped with a passive integrated transponder (PIT) tag (Biomark, Boise, United States), implanted subcutaneously in the flank. For small birds (~ 15 g), subcutaneous tags allow measuring the body temperature (T_b) as accurately as intra-peritoneal tags (Oswald et al., 2018a). In the aviaries, T_b was detected every 20 seconds by two PIT tag antennas (HPR plus, Biomark), placed next to the perches, during the six days of observations that occurred between 07-Dec-2018 and 17-Jan-2019 ($N = 331$ readings). We calculated the average T_b of each individual during the behavioural observation (using T_b values obtained within 5 min of each individual observation). Within sessions, there were 3.72 ± 0.44 T_b values on average per individual observation, and an individual T_b varied by $0.42 \pm 0.07^\circ\text{C}$ on average.

Time of Day

Because air temperature varied predictably throughout the day (Figure 1), to investigate time-of-day effects independently of T_{air} , we used time-of-day as a two-level variable, split at 2 pm when T_{air} typically plateaus. The two levels were thus: “midday” (11:00–14:00, ascending temperature phase, mean $T_{air} = 32.38^\circ\text{C}$, $N = 103$ sessions), and “afternoon” (14:00–17:00, descending phase, 32.41°C , $N = 79$ sessions).

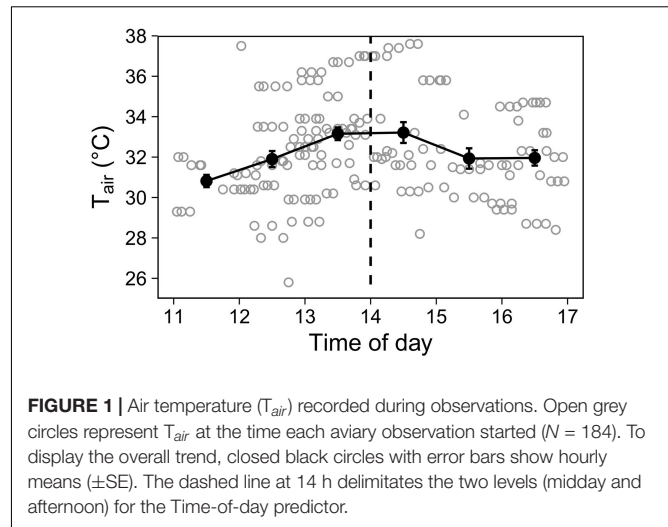


FIGURE 1 | Air temperature (T_{air}) recorded during observations. Open grey circles represent T_{air} at the time each aviary observation started ($N = 184$). To display the overall trend, closed black circles with error bars show hourly means ($\pm$ SE). The dashed line at 14 h delimitates the two levels (midday and afternoon) for the Time-of-day predictor.

Statistical Analyses

Analyses were performed separately on the unmanipulated cohort C_{cont} (without early-life predictors; $N = 30$ birds) on the one hand, and on the manipulated birds from C_{exp} (from the two replicate experiments; $N = 184$ birds) on the other hand, including playback and nest temperature as early-life predictors.

All statistical analyses were performed using R (version 4.0.1) via RStudio (RStudio Team, 2021). All models were fitted using the package *lme4* (Bates et al., 2021) and continuous predictors were normalised using the scale function, to facilitate estimate interpretation. Full model outputs are presented in the Supplementary Material (Supplementary Tables 1–3). We obtained the reduced models following a backward stepwise procedure, removing non-significant fixed factors, starting with interactions, until only significant or marginal ($p < 0.07$) terms remained (Crawley, 2012). We then used the Akaike's Information Criterion corrected for small sample sizes (AICc) to confirm the reduced models were the most parsimonious (i.e., lowest AICc).

Plasticity and Repeatability of Behaviour

The following analyses were restricted to individuals ($N = 103$) observed more than 10 times in total in C_{exp} , or more than 5 times in C_{cont} ($N = 23$), to ensure repeated observations per individual across the temperature range.

We tested whether panting response to microsite temperature at the cohort level was driven by between- and/or within-individual differences, following the method by van de Pol and Wright (2009) (used in e.g., Dingemanse et al., 2012; Hidalgo Aranzamendi et al., 2019). We used within-individual centering, to obtain two distinct predictors: between- and within-individual variation components. The “between-individual component” was calculated as the individual mean (i.e., average T_{loc} experienced by each individual across all its observations). The “within-individual component” was calculated, for each of an individual's observations, as the deviation from this individual mean (by subtracting individuals' average T_{loc} from each of their observed T_{loc}). We ran an initial binomial generalised linear mixed model

(GLMM), fitting panting behaviour as the response variable, and both the between- and within-individual components calculated above as predictors. Then, to test whether between- and within-individual variation differed significantly from each other, we ran a second GLMM including panting as the response variable and, as fixed effects, the within-individual component calculated above, and a predictor for the difference between the between- and within-individual effects (actually corresponding to T_{loc} in this model) (van de Pol and Wright, 2009).

Then, we tested for between-individual differences in the intercept (i.e., elevation) and slopes (i.e., plasticity) of the reaction norm of panting response to T_{loc} (Dingemanse et al., 2010). We fitted random regression models, including panting as the response variable, and the fixed effects as described below in full models (i.e., C_{cont} : T_{loc} , time-of-day, and sex; C_{exp} : T_{loc} , time-of-day, sex, prenatal playback (heat-calls or control-calls), nest temperature differential (T_{d-nest}), and playback by T_{loc} and playback by T_{d-nest} interactions). We included a random

TABLE 1 | Parameter estimates and 95% confidence intervals (CIs) of binomial generalised linear mixed models (GLMMs) using within-individual centering for panting behaviour, microsite use, and activity, in the control (C_{cont} ; $N = 226$ observations for 23 birds observed > 5 times) and experimental cohorts (C_{exp} ; $N = 1,929$ observations for 103 birds observed > 10 times).

Response variable	Cohort	Fixed effect	Est.	Lower 95% CI	Upper 95% CI	p-value
Panting	C_{cont}	Test within- and between-individual variation components				
		Intercept	-28.96	-46.76	-12.63	<0.001
		Within-individual T_{loc}	0.37	0.22	0.54	<0.001
		Between-individual T_{loc}	0.77	0.33	1.25	0.001
		Test difference between within- and between-individual components				
		Intercept	-28.96	-46.76	-12.63	<0.001
		Within-individual T_{loc}	0.37	0.22	0.54	<0.001
		Variance component difference	0.39	-0.06	0.87	0.100
	C_{exp}	Test within- and between-individual variation components				
		Intercept	-23.51	-31.08	-16.07	<0.001
		Within-individual T_{loc}	0.38	0.32	0.44	<0.001
		Between-individual T_{loc}	0.62	0.41	0.82	<0.001
		Test difference between within- and between-individual components				
		Intercept	-23.51	-31.08	-16.07	<0.001
		Within-individual T_{loc}	0.38	0.32	0.44	<0.001
		Variance component difference	0.24	0.03	0.45	0.023
Microsite use	C_{cont}	Test within- and between-individual variation components				
		Intercept	-19.55	-38.76	-1.60	0.038
		Within-individual T_{loc}	0.42	0.24	0.63	<0.001
		Between-individual T_{loc}	0.53	-0.02	1.11	0.065
		Test difference between within- and between-individual components				
		Intercept	-19.55	-38.76	-1.60	0.038
		Within-individual T_{loc}	0.42	0.24	0.63	<0.001
		Variance component difference	0.11	-0.46	0.70	0.704
	C_{exp}	Test within- and between-individual variation components				
		Intercept	-21.01	-28.70	-13.40	<0.001
		Within-individual T_{loc}	0.43	0.37	0.50	<0.001
		Between-individual T_{loc}	0.57	0.34	0.81	<0.001
		Test difference between within- and between-individual components				
		Intercept	-21.01	-28.70	-13.40	<0.001
		Within-individual T_{loc}	0.43	0.37	0.50	<0.001
		Variance component difference	0.14	-0.10	0.38	0.242
Activity	C_{cont}	Test within- and between-individual variation components				
		Intercept	4.05	-11.12	19.01	0.595
		Within-individual T_{loc}	-0.00	-0.15	0.15	0.989
		Between-individual T_{loc}	-0.13	-0.54	0.27	0.514
	C_{exp}	Test within- and between-individual variation components				
		Intercept	3.12	-4.47	10.67	0.419
		Within-individual T_{loc}	0.03	-0.03	0.09	0.339
		Between-individual T_{loc}	-0.12	-0.32	0.09	0.265

Bold indicates significant effects.

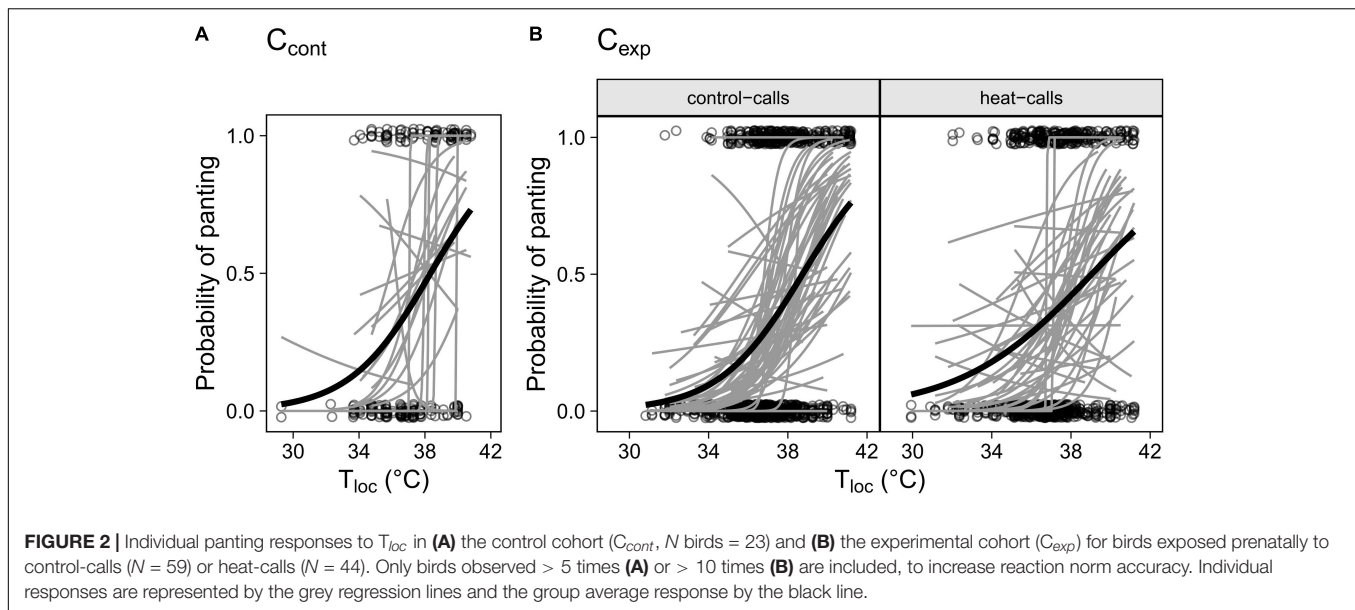


FIGURE 2 | Individual panting responses to T_{loc} in (A) the control cohort (C_{cont} , N birds = 23) and (B) the experimental cohort (C_{exp}) for birds exposed prenatally to control-calls (N = 59) or heat-calls (N = 44). Only birds observed > 5 times (A) or > 10 times (B) are included, to increase reaction norm accuracy. Individual responses are represented by the grey regression lines and the group average response by the black line.

intercept for date and, for bird identity (bird-ID), either a random intercept and slope, an intercept only, or no random effect. We used likelihood ratio tests (LRTs) to test the significance of the random terms. To test whether between-individual differences varied between the two playback groups, we also ran separate

TABLE 2 | Output of reduced binomial generalised linear mixed models (GLMMs) in the control cohort (C_{cont}), not subject to experimental procedures in early life.

Response variable	Fixed effects	Est.	SE	z	p-value
Microsite	as response to T_{air} ¹				
	Intercept	-2.51	0.38	-6.67	<0.001
	T_{air}	1.27	0.32	3.98	<0.001
Panting	as response to T_{air} ²				
	Intercept	-0.33	0.44	-0.74	0.460
	T_{air}	0.73	0.38	1.93	0.053
	microsite (floor)	-4.03	0.82	-4.90	<0.001
	as response to T_{loc} ³				
	Intercept	-0.32	0.50	-0.64	0.524
Activity	T_{loc}	1.52	0.31	4.87	<0.001
	time (afternoon)	-1.03	0.40	-2.58	0.010
	as response to T_{air} ²				
	Intercept	-1.22	0.29	-4.21	<0.001
	microsite (floor)	0.90	0.43	2.08	0.037
	as response to T_{loc} ³				
	Intercept	-1.37	0.34	-4.03	<0.001
	time (afternoon)	0.54	0.34	1.61	0.109

Outputs for microsite use, panting behaviour and activity, as a response to either air temperature (T_{air}) or microsite temperature (T_{loc}), with also sex, time of day and microsite as additional predictors. N_{obs} = 252 for 30 birds.

¹Full model: $microsite \sim sex + time + T_{air} + (1|bird-ID) + (1|date)$.

²Full model: $response \sim microsite + T_{air} + microsite \times T_{air} + (1|bird-ID) + (1|date)$.

³Full model: $response \sim sex + T_{loc} + time + (1|bird-ID) + (1|date)$.

Significant effects are indicated in bold font.

models for birds exposed to control- or heat-calls. Since not all individuals were observed in all sessions, there was variation in the number of observations per individual (C_{cont} : mean = 10.66, range = 6–17; C_{exp} : mean = 22.79, range = 11–47) and the range of temperatures at which each individual was observed (C_{cont} : min T_{loc} range = 2.8°C, max T_{loc} range = 10.71°C; C_{exp} : min range = 3.37°C; max range = 10.58°C). We used simulated datasets to demonstrate that this sampling design was not creating spurious inter-individual variation, nor masking variation (see results and **Supplementary Material, Figure 1**). As we found variation in intercept but not slope for bird identity (see section “Results”), all models below included date and bird-ID as random intercepts (unless otherwise specified).

For microsite use and activity, we also applied the within-individual centering method described above to test for between- and/or within-individual differences in microsite use across air temperature (T_{air}), and activity across T_{loc} .

Microsite Use

To test how the birds used the microsites, in C_{cont} , we ran binomial GLMMs with microsite (floor or perches) as a response variable and T_{air} , time-of-day and sex as fixed factors. For C_{exp} , we added as fixed factors the prenatal playback (heat-calls or control-calls), nest temperature differential (T_{d-nest}) and the two-way interactions between playback and temperature during observation (T_{air}) or development (T_{d-nest}).

Panting Behaviour and Activity

For each of the two cohorts, we ran two binomial GLMMs, with either panting or activity as a response variable. First, we assessed how panting or activity varied with microsite, T_{air} , and their interaction, in both cohorts. Then, because microsites differed in temperature (by 4.9°C on average), to test how birds responded to the actual temperature they were experiencing at their location in the aviary, we ran another two models per cohort (for panting and activity respectively) using T_{loc} instead of T_{air} . For C_{cont} , we

used T_{loc} , time-of-day and sex as predictors. For C_{exp} , as above, we added as predictors the prenatal playback, T_{d-nest} and the two-way interactions between playback and T_{loc} , playback and T_{d-nest} , and, T_{d-nest} and T_{loc} . Using a three-level variable for activity (resting, perching, active) in a Cumulative link mixed model [CLMM; package *ordinal*, (Christensen, 2019)] led to the same results as using the binomial GLMM; therefore only the latter is presented.

Body Temperature

To establish whether, at moderate microsite temperature (T_{loc}), panting is indicative of a lesser ability to maintain low T_b , or instead reflects a higher investment in respiratory evaporative water loss to prevent T_b rise, we assessed variation in T_b in a subset of birds equipped with thermosensitive PIT tags ($N = 23$). We ran a GLMM with T_b as a response variable, and T_{loc} , panting (absence/presence) and their interaction as predictors, including bird-ID as a random intercept and using a Gamma distribution and a log-link function. We did not include observation date ($N = 6$ days) as a random effect, due to extremely low variance. To allow meaningful interpretation, we restricted this analysis to when $T_{loc} < \text{minimal } T_b$ (i.e., 40°C). Sample sizes were insufficient to also investigate T_b at $T_{loc} > 40^\circ\text{C}$. Data was transformed by subtracting the minimal T_b in the dataset from all T_b values and adding a constant of 1 to keep only meaningful variation and obtaining positive values to fit the Gamma distribution.

RESULTS

Plasticity and Repeatability of Behaviour

In both control (C_{cont}) and experimental (C_{exp}) cohorts, the panting response to microsite temperature (T_{loc}) was due to both phenotypic differences between individuals and behavioural flexibility within individuals. Specifically, some individuals consistently panted more than others (between-individual component, **Table 1**), and, each individual also panted more when T_{loc} increased (within-individual component, **Table 1**). The variation between individuals was significantly larger than that within, in C_{exp} , but not in C_{cont} (differences between variation components, **Table 1**).

Individuals differed significantly in the intercept of their panting response (i.e., elevation) but not in the reaction norm slope (**Figure 2**). This indicates that individuals varied in the temperature threshold at which they started panting, but generally not in how panting increased with temperature past that threshold. This was true in both C_{cont} (**Figure 2A**; LRTs: elevation, $\chi^2 = 7.63$, $p = 0.006$; slope: $\chi^2 = 4.72$, $p = 0.094$) and C_{exp} (LRTs: elevation, $\chi^2 = 80.50$, $p < 0.001$; slope: $\chi^2 = 1.07$, $p = 0.586$), including when control- and heat-call birds were considered separately: intercepts varied in both playback groups (LRTs: $\chi^2 = 35.51$, $p < 0.001$ and $\chi^2 = 42.69$, $p < 0.001$ respectively) but control-call birds were remarkably consistent in slope (**Figure 2B**, $\chi^2 = 0.06$, $p = 0.968$) while heat-call birds showed slightly more variation, even though non-significant (**Figure 2B**; $\chi^2 = 2.57$, $p = 0.277$).

Our sample size and sampling design, including within playback groups, were nonetheless suitable to detect inter-individual variation in slope, had it been stronger (see simulations in **Supplementary Material**).

Similarly, for microsite use, variation in response to air temperature (T_{air}) was explained by the combination of inter-individual phenotypic differences (although only marginally in C_{cont}) and within-individual behavioural flexibility (**Table 1**).

TABLE 3 | Output of reduced binomial GLMMs in the experimental cohort (C_{exp}), subject to experimental manipulation of prenatal playback (control-calls or heat-calls) and postnatal nest temperature differential (T_{d-nest}).

Response variable	Fixed effects	Est.	SE	z	p-value
Microsite	as response to T_{air} and developmental conditions ¹				
	Intercept	-3.98	0.56	-7.07	<0.001
	T_{air}	0.93	0.21	4.37	<0.001
	time (afternoon)	-1.01	0.19	-5.22	<0.001
	sex (male)	0.75	0.18	4.24	<0.001
	prenatal playback (heat-calls)	0.00	0.18	0.00	0.998
	T_{d-nest}	-0.28	0.11	-2.43	0.015
Panting	as response to T_{air} ²				
	Intercept	-0.76	0.24	-3.11	0.002
	T_{air}	0.84	0.12	6.86	<0.001
	microsite (floor)	-1.90	0.20	-9.45	<0.001
	as response to T_{loc} and developmental conditions ³				
	Intercept	-0.80	0.24	-3.30	0.001
	T_{loc}	1.05	0.10	10.66	<0.001
Activity	time (afternoon)	-0.54	0.14	-3.90	<0.001
	prenatal playback (heat-calls)	-0.01	0.17	-0.05	0.960
	T_{d-nest}	0.15	0.08	1.99	0.047
	playback x T_{loc}	-0.29	0.12	-2.45	0.014
	as response to T_{air} ²				
	Intercept	-1.42	0.18	-7.95	<0.001
	microsite (floor)	-0.45	0.17	-2.64	0.008
	as response to T_{loc} and developmental conditions ³				
	Intercept	-1.66	0.23	-7.28	<0.001
	T_{loc}	0.24	0.07	3.52	<0.001
	time (afternoon)	-0.39	0.14	-2.84	0.004
	sex (male)	0.30	0.12	2.44	0.015
	prenatal playback (heat-calls)	0.37	0.12	3.02	0.003
	T_{d-nest}	-0.11	0.06	-1.86	0.063

Outputs for microsite use, panting behaviour and activity as a response to either air (T_{air}) or microsite temperature (T_{loc}), with also sex, time of day, microsite, playback and T_{d-nest} as additional predictors. $N_{obs} = 2,402$ for 184 birds.

¹Full model: $\text{microsite} \sim T_{air} + \text{time} + \text{sex} + \text{prenatal playback} + T_{d-nest} + \text{playback} \times T_{air} + \text{playback} \times T_{d-nest} + (1 | \text{bird-ID}) + (1 | \text{date})$.

²Full model: $\text{response} \sim \text{microsite} + T_{air} + \text{microsite} \times T_{air} + (1 | \text{bird-ID}) + (1 | \text{date})$.

³Full model: $\text{response} \sim T_{loc} + \text{time} + \text{sex} + \text{prenatal playback} + T_{d-nest} + \text{playback} \times T_{loc} + \text{playback} \times T_{d-nest} + T_{d-nest} \times T_{loc} + (1 | \text{bird-ID}) + (1 | \text{date})$.

Significant effects are indicated in bold font.

Unlike for panting response however, the within-individual variation was as large as that between individuals (i.e., difference between variation components, **Table 1**).

Lastly, for activity, there was no significant between- nor within-individual variation (**Table 1**), consistent with only a weak effect of T_{loc} (see below), in both C_{cont} and C_{exp} .

Microsite Use

During the observations, the floor was on average 4.9°C cooler than the perches (ranging from 3.6 to 5.8°C). In both cohorts, birds were more likely to be on the floor as T_{air} increased (**Tables 2, 3** and **Figure 3A**). In addition, in the experimental cohort C_{exp} , the probability of being on the floor was higher at midday than in the afternoon, and in males (**Table 3** and **Figures 3B,C**).

Early-life experience also affected microsite use. There was a significant interaction between the prenatal playback and nest temperature differential (T_{d-nest} ; **Table 3** and **Figure 3D**): in control-call birds, as expected, individuals reared in cooler nests were more likely to use the floor than those from hotter nests. However, the opposite was true in birds exposed to prenatal heat-calls.

Panting Response

As expected given its thermoregulatory function, panting probability increased with environmental temperature. Panting increased with T_{air} [although $p = 0.053$ for C_{cont} : **Table 2**; C_{exp} : **Table 3**], and was significantly higher on the perches than on the floor (**Tables 2, 3** and **Figure 4A**). Correspondingly, the probability of panting increased with T_{loc} (i.e., temperature at the bird's location) in both cohorts (**Tables 2, 3**). In addition, birds panted significantly more, earlier in the day (**Tables 2, 3** and **Figure 4B**).

Regarding early-life conditions, panting probability increased slightly but significantly with T_{d-nest} (**Table 3** and **Figure 4C**), but there was no interaction between T_{d-nest} and T_{loc} (**Supplementary Table 2**). Additionally, there was a significant interaction between the prenatal playback and T_{loc} (**Table 3** and **Figure 4D**): panting probability increased with T_{loc} in both playback groups; however, this increase was sharper in control-call birds, as heat-call birds started panting at lower T_{loc} but panted less than controls at high T_{loc} . This effect was robust, as it was also visible in the raw data when panting observations were divided up into temperature categories (**Figure 4E**). This difference between playback groups was not merely explained

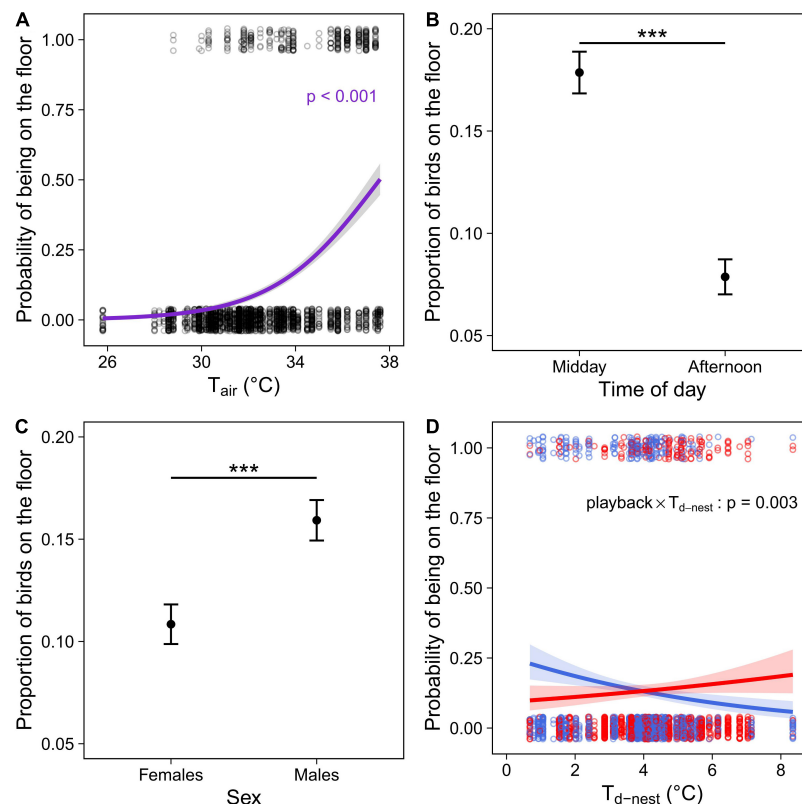


FIGURE 3 | Microsite use (floor versus perches in outdoor aviary) in the experimental cohort (C_{exp}) comprising individuals that were exposed to a prenatal playback of either heat-calls or control-calls and reared under different nest temperature differentials (T_{d-nest} ; $N_{obs} = 2402$ for 184 birds). **(A)** Probability of birds being on the floor depending on the air temperature during observation (T_{air}); proportion of birds on the floor depending on **(B)** the time of day or **(C)** sex; and **(D)** probability of using the floor as a function of the thermal microenvironment experienced as a nestling (°C above air in the nest), in heat-call (red, $N = 1055$ observations for 84 birds) or control-call birds (blue, $N = 1347$ for 100 birds). Open circles show each observation, solid circles with error bars **(B,C)** represent the mean $\pm$ SE. Regression lines **(A,D)** are shown with 95% CIs. *** $p < 0.001$.

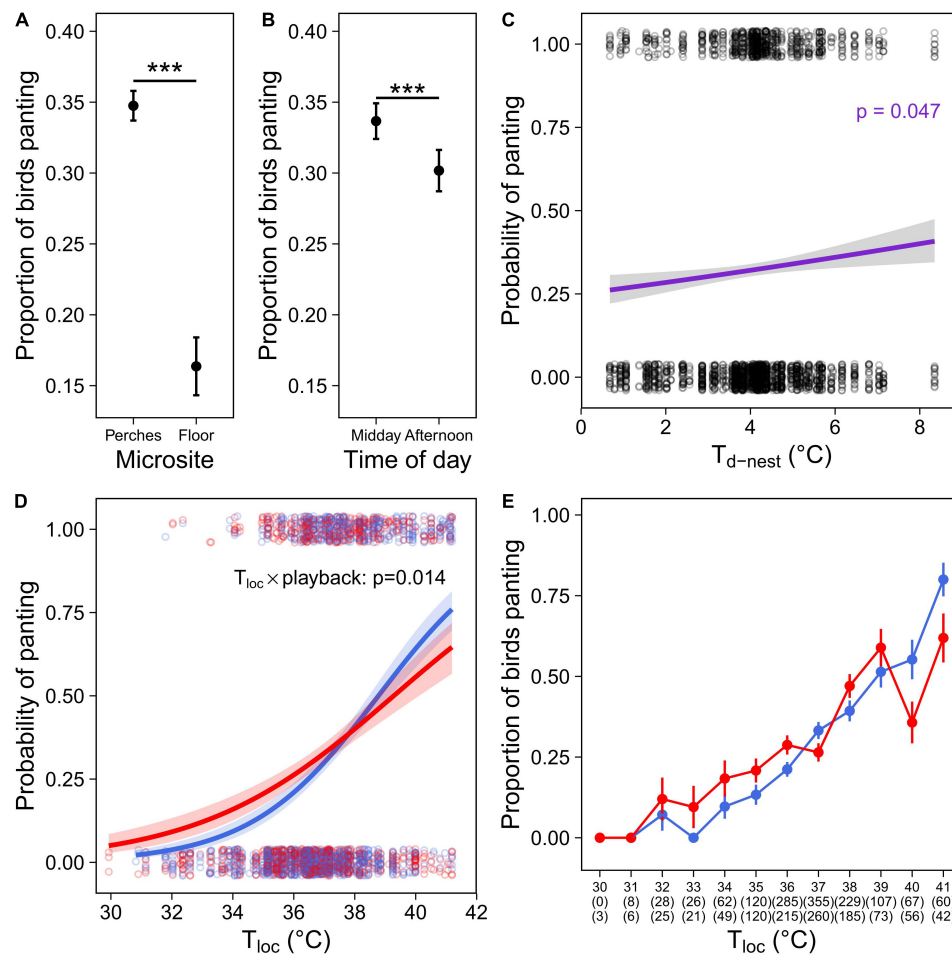


FIGURE 4 | Panting behaviour in the experimental cohort (C_{exp}), comprising individuals that were exposed to a prenatal playback of either heat-calls or control-calls and reared under different nest temperature differentials (T_{d-nest} ; $N_{obs} = 2,402$ for 184 birds). Proportion of birds panting depending on (A) microsite use; or (B) the time of day. (C) Probability of panting as function of the thermal microenvironment experienced as a nestling ($^{\circ}\text{C}$ above air in the nest). Panting response to T_{loc} as: (D) Probability of panting over T_{loc} or (E) proportion of birds panting (mean per one-degree class $\pm$ SE) over T_{loc} , in heat-calls (red, $N = 1,055$) or control-calls birds (blue, $N = 1,347$). For panel (D), the interaction between prenatal playback and T_{loc} remains significant without the three observations at 30°C ($p = 0.0013$). For panel (E), numbers in brackets indicate sample sizes (i.e., number of birds observed) per temperature class and playback group: control-calls (top row) or heat-calls (bottom row). Open circles show each observation, solid circles with error bars represent means $\pm$ SE. Regression lines are shown with 95% CIs. *** $p < 0.001$.

by their differential microsite use (reported above), since this interaction remained significant when birds on the floor were excluded ($p = 0.006$, **Supplementary Table 4**).

At environmental temperature below normothermic body temperature (i.e., $T_{loc} < 40^{\circ}\text{C}$), body temperature (in a subset of birds equipped with PIT tags) increased significantly with T_{loc} , in both panting and non-panting individuals (**Table 4** and **Figure 5**). T_b was nonetheless not higher in panting than non-panting birds ($T_b = 41.39^{\circ}\text{C}$ versus 41.42°C respectively, **Figure 5**). This suggests that, at $T_{loc} < 40^{\circ}\text{C}$, higher panting activity was not indicative of a lesser ability to maintain normothermic T_b .

Activity

In C_{exp} , birds were significantly less active on the floor than on the perches (**Table 3**; $N = 2402$ observations), although the reverse was true in the smaller sample of C_{cont} (**Table 2**; $N = 252$

observations). Birds in C_{exp} were also more active at midday than in the afternoon (**Table 3**) and, surprisingly, they were slightly but significantly more active at higher T_{loc} (**Table 3**). Additionally, males were more active than females in C_{exp} (**Table 3**).

Furthermore, in C_{exp} , birds exposed to prenatal heat-calls were more active than those exposed to control-calls (**Table 3** and **Figure 6A**), and so across the temperature gradient (**Table 3** and **Figure 6B**). Lastly, birds reared in hotter nests were marginally less active than those reared in cooler nests ($p = 0.063$ in reduced model: **Table 3**; $p = 0.018$ in full model: **Supplementary Table 2**).

DISCUSSION

We showed for the first time in endotherms that inter-individual variation in behavioural thermoregulation at adulthood can originate from early-life experience. Indeed, individuals

TABLE 4 | Output of main effects from a GLMM in a subset of birds equipped with PIT tags, to measure body temperature (T_b), as function of panting behaviour and microsite temperature (T_{loc}), up to $T_{loc} = 40^\circ\text{C}$.

Response variable	Fixed effects	Est.	SE	t	p-value
T_b^1	Intercept	0.91	0.05	17.05	< 0.001
	T_{loc}	0.07	0.02	3.37	< 0.001
	Panting	-0.06	0.06	-1.00	0.319

$N = 105$ behavioural observations for 23 birds.

¹ Full model: $T_b \sim T_{loc} + \text{panting} + \text{panting} \times T_{loc} + (1 | \text{bird-ID})$.

Significant effects are indicated in bold font.

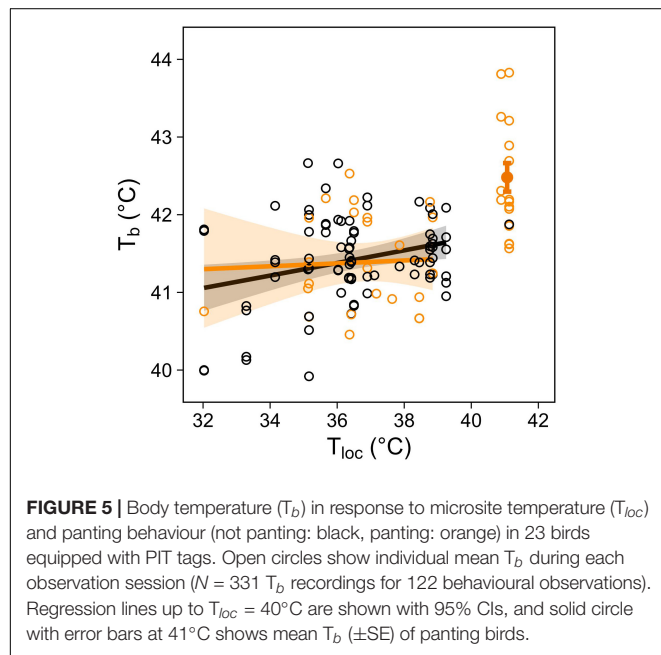


FIGURE 5 | Body temperature (T_b) in response to microsite temperature (T_{loc}) and panting behaviour (not panting: black, panting: orange) in 23 birds equipped with PIT tags. Open circles show individual mean T_b during each observation session ($N = 331$ T_b recordings for 122 behavioural observations). Regression lines up to $T_{loc} = 40^\circ\text{C}$ are shown with 95% CIs, and solid circle with error bars at 41°C shows mean T_b ($\pm\text{SE}$) of panting birds.

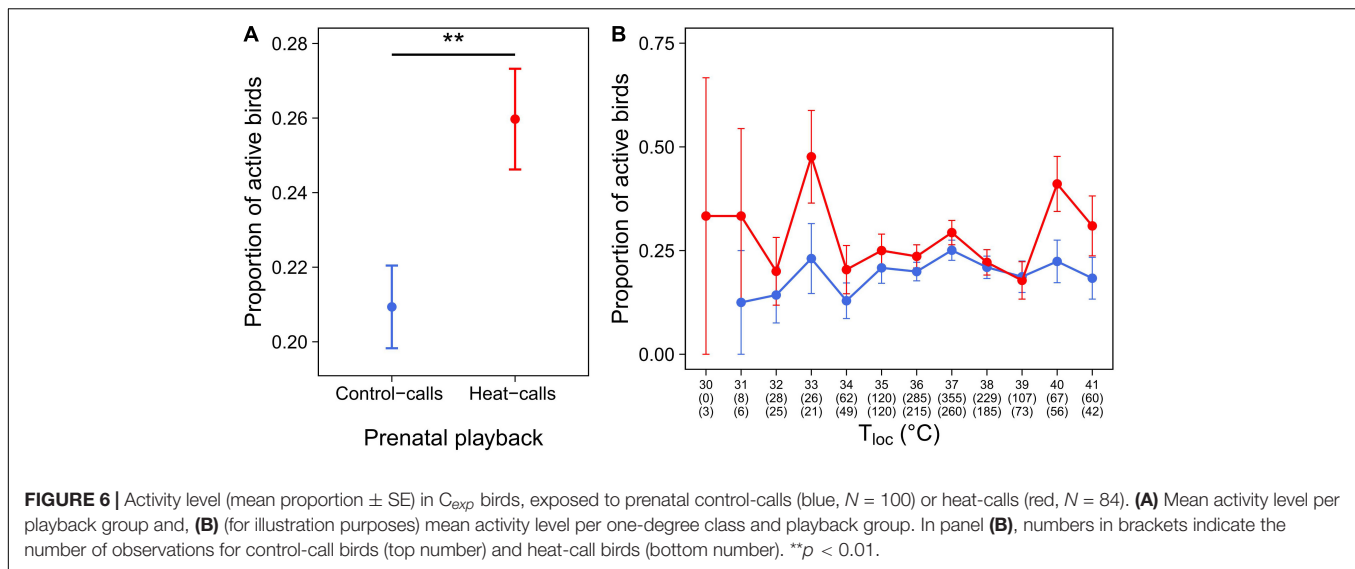
consistently varied in their thermoregulation strategy, with some individuals starting panting at lower air temperature than others (i.e., differences in intercept). Prenatal acoustic experience affected panting, as well as both other behavioural responses tested. Namely, birds exposed to heat-calls started panting at lower temperatures than control birds, but panted less at high temperatures, suggesting different heat-regulation strategies. We propose that such strategy in heat-call birds may improve water saving at high temperature extremes and/or allow maintaining high activity levels, since heat-call birds were also more active than controls, across the gradient of summer temperatures. Lastly, effects on microsite use were more complex, as it varied with the interaction of early acoustic and thermal experiences. Overall, our study reveals that a prenatal acoustic signal of heat can shape how individuals adjust behaviourally to thermal challenges at adulthood. This, in turn, has the potential to alter the selection pressures individuals are exposed to, and thereby the fitness components affected by high temperatures.

Early-life conditions, and most remarkably, prenatal acoustic experience, affected individual behavioural responses to heat on the long-term, with potential fitness consequences. Heat-call

birds panted less at high temperatures (T_{loc}), but more in cooler conditions, compared to control-call birds. Given the significant water loss associated with panting, moderating panting may correspond to a water-saving strategy. In arid environments, where birds may need to fly several kilometres to scarcely distributed drinking water, saving water is likely most crucial at very high temperatures, when flight may lead to excessive metabolic heat production. When environmental temperature rises to the level of normothermic body temperature (c.a. 40°C), small passerines often use hyperthermia (i.e., let T_b rise above normothermia) to save water and energy by reducing investment in thermoregulation (Tieleman and Williams, 1999; Gerson et al., 2019). It is therefore possible that heat-call individuals rely more on hyperthermia at high environmental temperature ($>40^\circ\text{C}$) when water may be less readily accessible (in the wild), but pant more in less extreme conditions, when panting does not incur severe dehydration or hyperthermia risk. Importantly, body temperature was not higher in panting than non-panting birds, which suggests that panting intensity, rather than a sign of higher heat-stress, may reflect differential thermoregulation strategies, in which the reliance on respiratory evaporative water loss versus alternative thermoregulatory pathways varies. The benefits of higher panting activity, also observed overall in birds from hotter nests, remain to be established. However, they may include reducing heat-load accumulation, or maintaining activity. Indeed, heat-call birds remained more active across the range of warm to high temperatures. If the activity levels we measured in our experiment reflect individual ability to maintain foraging and breeding activity in the heat, heat-call exposure may increase reproductive success in summer conditions, as previously demonstrated (Mariette and Buchanan, 2016). Overall, our results suggest that prenatal heat-call exposure did not reduce overall thermoregulatory needs, but instead altered individual panting strategy to better balance water loss and/or maintain higher activity levels. Our findings thus shed light on how acoustic developmental programming may impact life-long fitness by shaping individual behavioural responses to hot conditions.

As expected, and documented in other species (Smit et al., 2013; Cunningham et al., 2015; Pattinson and Smit, 2017; Xie et al., 2017; Oswald et al., 2019), panting and use of cooler microsites (here, the floor) increased with temperature (measured as T_{air} or T_{loc}). In addition, we found that, after controlling for microsite temperature, birds panted more, earlier in the day. Such diurnal patterns have also been observed in other species for panting (Grant, 1982; Smit et al., 2013) as well as for heat-calling in zebra finches [associated with higher water loss; (Mariette et al., 2018; Pessato et al., 2020)]. Nonetheless, we also showed that cooler microsite use was higher earlier in the day, which to our knowledge had not been shown elsewhere. Higher panting might occur in anticipation of air temperature peaking in late afternoon, or to sustain higher activity, which was also higher than in the afternoon.

Importantly, our aviary observations seem to mirror expected behavioural patterns in wild birds. Indeed, birds on the floor were less active than individuals remaining on perches (at least in the experimental cohort), similarly to studies in free living



birds where individuals either cease activity and seek thermal refuges, or remain active in exposed sites (Smit et al., 2016; Pattinson and Smit, 2017). Interestingly, birds on the floor were also half as likely to pant as those on the perches (16 versus 35% respectively). This points to the occurrence of two alternative behavioural strategies where individuals save water and energy by reducing heat production (activity) and exposure to heat (microsite), or maintain normal behavioural activity at the cost of higher thermoregulatory needs, and thus higher energy and water expenditure. That both phenotypes were expressed even though birds had access to *ad libitum* food and water in the aviaries is interesting. This might indicate that behavioural responses are not as flexible as commonly assumed, perhaps as a consequence of being dictated by physiological capacities. This is in agreement with our findings that individuals consistently varied in their panting responses [and specifically in their panting threshold (estimated by random intercept)], and that such response was partly determined by early-life effects.

In conclusion, our study shows that early-life conditions have long-term effects on behavioural thermoregulatory strategies, contributing therefore to consistent inter-individual differences at adulthood. This study also reveals the role of prenatal sound in shaping individuals' behavioural responses to thermal changes. These responses likely reflect differential trade-off modulation between thermoregulation, water balance and activity, and may explain long-lasting positive effects of heat-call exposure on reproductive fitness. Overall, our findings bring us closer to understanding how behavioural flexibility alters selection pressures by high temperatures, which is essential to predict the impact of anthropogenic climate change on species persistence.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Deakin University Animal Ethics Committee (project G23-2018).

AUTHOR CONTRIBUTIONS

EU and MM designed the study, and wrote the manuscript. MM conducted the prenatal playback experiments. EU collected, processed and analysed behavioural data with help from MM. Both 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.2022.818278/full#supplementary-material>

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In the Hot Seat: Behavioral Change and Old-Growth Trees Underpin an Australian Songbird's Response to Extreme Heat

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Anthropogenic climate change is increasing the frequency and intensity of heat waves, thereby threatening biodiversity, particularly in hot, arid regions. Although free-ranging endotherms can use behavioral thermoregulation to contend with heat, it remains unclear to what degree behavior can buffer organisms from unprecedented temperatures. Thermoregulatory behaviors that facilitate dry heat loss during moderate heat become maladaptive once environmental temperatures exceed body temperature. Additionally, the costs associated with behavioral thermoregulation may become untenable with greater heat exposure, and effective cooling may be dependent upon the availability of specific microhabitats. Only by understanding the interplay of these three elements (responses, costs and habitat) can we hope to accurately predict how heat waves will impact wild endotherms. We quantified the thermoregulatory behaviors and microhabitat use of a small passerine, the Jacky Winter (*Microeca fascians*), in the mallee woodland of SE Australia. At this location, the annual number of days $\geq 42^{\circ}\text{C}$ has doubled over the last 25 years. The birds' broad repertoire of behavioral responses to heat was nuanced and responsive to environmental conditions, but was associated with reduced foraging effort and increased foraging costs, accounting for the loss of body condition that occurs at high temperatures. By measuring microsite surface temperatures, which varied by up to 35°C at air temperatures $> 44^{\circ}\text{C}$, we found that leaf-litter coverage and tree size were positively correlated with thermal buffering. Large mallee eucalypts were critical to the birds' response to very high temperatures, providing high perches that facilitated convective cooling, the coolest tree-base temperatures and the greatest prevalence of tree-base crevices or hollows that were used as refuges at air temperatures $> 38^{\circ}\text{C}$. Tree-base hollows, found only in large mallees, were cooler than all other microsites, averaging 2°C cooler than air temperature. Despite the plasticity of the birds' response to heat, 29% of our habituated study population died when air temperatures reached a record-breaking 49°C , demonstrating the limits of behavioral thermoregulation and the potential vulnerability of organisms to climate change.

Keywords: behavioral thermoregulation, heat waves, heat dissipation, microhabitat, thermal profile, avian thermoregulation, large mallee trees, thermal refuge

INTRODUCTION

Anthropogenic climate change has led to an increase in the frequency and intensity of extreme weather events, particularly heat waves (Stillman, 2019). Record-breaking temperatures are having severe consequences for biodiversity, with mass mortality events (Welbergen et al., 2008; McKechnie and Wolf, 2010; McKechnie et al., 2021), the loss of arid-zone communities (Riddell et al., 2019) and reduced population viability (Thomas et al., 2006; Ruthrof et al., 2018; Stillman, 2019).

Physiologically informed models predict severe losses for birds in hot, arid environments due to acute heat stress (Conradie et al., 2020), but they do not take into account the positive effect of many thermoregulatory behaviors, which may be substantial based on the few studies that have evaluated the survival of wild individuals during heatwaves (Cooper et al., 2019; Sharpe et al., 2019). At high temperatures, behavioral thermoregulation serves two functions: the minimization of heat gain and the facilitation of heat dissipation. Given that solar radiation is the primary source of heat load (Mitchell et al., 2018), with 55% of the sun's energy comprised of non-visible radiant heat (Stuart-Fox et al., 2017), avoiding sun exposure through shade use and altered activity time budgets is an almost universal behavioral response (Hetem et al., 2012; Hall and Chalfoun, 2019). However, this is only a partial solution because reflected solar radiation and radiant heat emanating from sun-warmed surfaces also contribute to heat load. Air temperatures that exceed body temperature also result in heat gain, which endotherms avoid by using cooler microhabitats (Williams et al., 1999; Walde et al., 2009; Carroll et al., 2015; Ruth et al., 2020) but this strategy relies upon thermal heterogeneity within the organism's environment. Behaviors that curtail metabolic heat production, such as inactivity and fasting, also reduce heat gain (Beale et al., 2018) but may adversely impact energy balance (Youngentob et al., 2021).

At high environmental temperatures, endotherms must dissipate heat. They can do this *via* sensible (dry) heat transfer or latent (evaporative) heat transfer. All forms of dry heat transfer (conduction, convection and radiation) depend upon the existence of a temperature gradient between the surface of the animal and its environment: the steeper the gradient, the more effective the heat transfer. Conductive cooling occurs when objects of differing temperature touch, and it is enhanced by maximizing bodily contact with cool, poorly insulated substrates, such as sprawling in newly excavated sand, hugging bare tree trunks or clasping cool perches (Dawson, 1973; Muiruri and Harrison, 1991; Briscoe et al., 2014). Convective heat transfer occurs *via* the currents within a fluid or gas (e.g., air) which are generated by temperature-related differences in the medium's density. Losing heat *via* convection is impaired by an endotherm's plumage or pelage and tends to be ineffectual unless the velocity of the air flow is artificially increased, by wind or rapid movement (i.e., "forced" convective cooling; Mitchell et al., 2018). Long, narrow objects and those with a large surface area to mass ratio, lose heat more rapidly (Mitchell et al., 2018), so animals can maximize convective cooling by altering their posture and orientation, reducing the insulative properties of

their fur or feathers by ptilo- or piloretraction, and by exploiting windy microsites. In radiant heat transfer, animals lose heat by emitting infrared electromagnetic waves. Although mechanisms that maximize radiant heat loss are usually under autonomic control (e.g., the vasodilation of capillaries in uninsulated body parts such as beaks, ears, legs or facial skin; Tattersall et al., 2009; Weissenböck et al., 2010), the efficiency of these thermal windows can be enhanced behaviorally *via* movement (e.g., ear flapping), changes in posture and orientation, and by microsite choice.

Once environmental temperatures equal or exceed body temperature, interactive strategies that facilitate sensible (dry) cooling will become detrimental, serving to expediate the acquisition of heat instead. Although endotherms can use regulated hyperthermia (allowing body temperature to rise above normal levels) to maximize and prolong the efficiency of dry cooling mechanisms (Tieleman and Williams, 1999), there is a limit to how high body temperature can safely rise (McKechnie and Wolf, 2019). When environmental temperature exceeds body temperature, organisms must resort to evaporative (or latent) cooling to prevent lethal heat stress. Behaviors that facilitate evaporative cooling include bathing, mud-wallowing, licking saliva on the body and urohidrosis (using excreta to wet parts of the body), while the primary physiological mechanisms are panting, sweating in some mammals, or gular fluttering in some birds (Ingram, 1965; Dawson, 1973; Arad et al., 1989; Mole et al., 2016; McKechnie and Wolf, 2019). Rates of evaporative cooling can be accelerated by behaviors that enhance convective heat loss, but, for animals with limited access to water, the loss of body water poses a serious dehydration risk (Conradie et al., 2020). During prolonged heat exposure, behavioral strategies that constrain evaporative water loss to levels required for thermal homeostasis will aid survival, and animals may thus seek out microsites with lower wind velocity and greater humidity (Walde et al., 2009; Luna et al., 2020).

Although we know that endotherms can adopt a wide array of thermoregulatory behaviors, the breadth of individual species' behavioral repertoires are largely unknown, especially in small species, and it is unclear to what degree responses to heat are flexible or fixed. The extent to which organisms can modify their thermoregulatory behavior under global warming is critical, given that behavioral adjustment can buffer organisms from lethal effects and provide time for genetic adaptations to evolve (Tuomainen and Candolin, 2011). There are, however, limitations. Firstly, an organism's behavioral repertoire is the product of selection pressures generated by past environments, so its responses may be insufficient, or simply inappropriate, to meet novel conditions (Tuomainen and Candolin, 2011). Many thermoregulatory behaviors cannot simply be "scaled up" in response to increasing temperature because behaviors that facilitate dry heat loss in moderate heat are maladaptive (promoting heat gain) once environmental temperatures become extreme. Additionally, the costs associated with thermoregulatory behaviors, such as dehydration risk from panting or sweating, or loss of body condition from changed foraging patterns, may become untenable with greater heat exposure (Albright et al., 2017; Cunningham et al., 2021; Youngentob et al., 2021). Finally, many thermoregulatory

behaviors are dependent upon the thermal heterogeneity of an organism's environment, yet anthropogenic environmental change can alter the thermal profile of habitat, reducing the availability of critical thermally buffered microsites (Chen et al., 1999; Neel and McBrayer, 2018). Only by obtaining a clear understanding of how organisms respond behaviorally to heat, the costs associated with these behaviors and which elements of habitat are essential for these behaviors, will we be able to effectively predict the impact of heat waves and begin to develop appropriate mitigation strategies.

This study focusses on a small passerine, the Jacky Winter (*Microeca fascians*), living in a semi-arid region of southern Australia experiencing unprecedented heat wave events (Figure 1). Prior work at the study site found that wild individuals (with no access to water) can withstand > 10 h exposure to air temperatures $\geq 40^{\circ}\text{C}$, even though we would expect them to succumb to lethal dehydration based on the thermal tolerance of similar sized passerines in the laboratory (Sharpe et al., 2019). The aim of this study was to determine the behavioral strategies the birds use and identify factors that may limit their ability to cope with rising environmental temperatures in the future. We quantified the breadth of the birds' repertoire of thermoregulatory behaviors, from mild to

near-lethal temperatures, to assess whether their responses were nuanced, flexible and responsive to environmental conditions. We hypothesized that at high air temperatures the birds would adopt heat dissipation behaviors, such as wing-spreading and panting, reduce high intensity motor behaviors and alter their foraging time budget to avoid feeding. We predicted that increasing air temperature would lead to changes in microhabitat use, with potential shifts in perch height, shade use and the size of perch trees. We identified the potential costs of these temperature-related changes in behavior, in relation to foraging effort. We also examined to what degree the birds' thermoregulatory behaviors were dependent on particular microhabitats and quantified the associated thermal benefits of these microsites. Without a detailed mechanistic understanding of how small endotherms are utilizing habitat during heat waves, the costs involved, and which components of the habitat serve as critical thermal buffers, it is impossible to accurately predict the impact of climate change on population persistence or devise conservation strategies that protect vulnerable biodiversity.

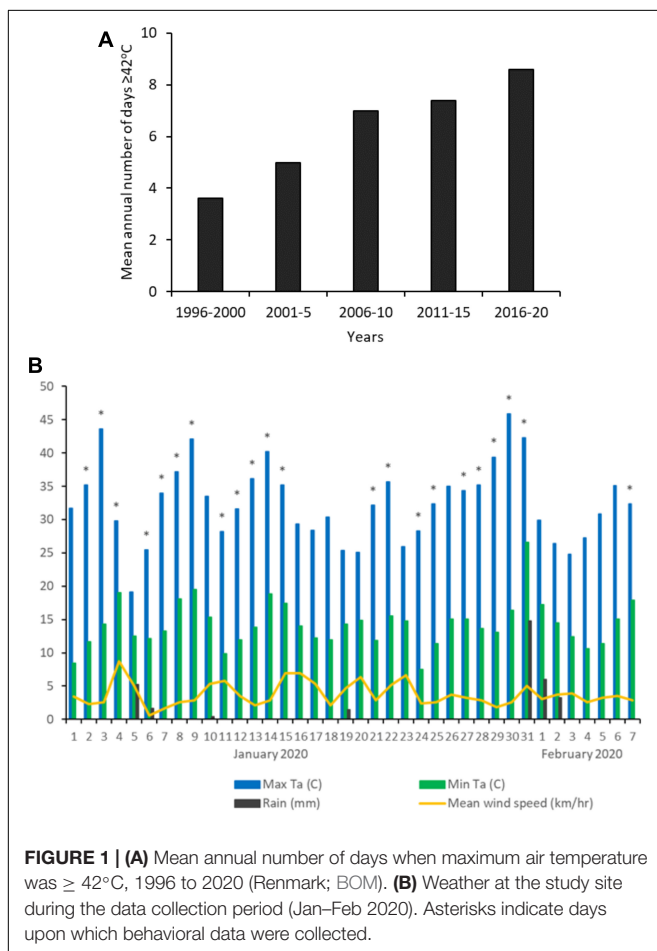
MATERIALS AND METHODS

Study Site

The study was undertaken in semi-arid mallee woodland at Calperum Station, South Australia ($34^{\circ}03'S$ $140^{\circ}38'E$) during the austral summer (January–February) of 2020. The vegetation at the study site is comprised of low, multi-stemmed mallee eucalypts (*Eucalyptus oleosa*, *E. dumosa*, *E. incrassata*, *E. socialis*) and sugarwood (*Myoporum platycarpum*) above a sparse understorey of *Senna* and *Acacia* shrubs and porcupine grass (*Triodia scariosa*). All study groups lived in woodland that had not been burnt within the last 50 years. Mean annual rainfall is 251 mm, distributed irregularly across the year, and mean daily maximum temperatures in January and February are 33.8 and 32.7°C , respectively [data for Renmark, 1996–2020; Bureau of Meteorology, Australia (BOM)]. The frequency of extreme heat events at this location has been increasing, with the annual number of days $\geq 42^{\circ}\text{C}$ more than doubling in the last 25 years (Figure 1A; Renmark, BOM).

Study Species

The study species—the Jacky Winter (*Microeca fascians*)—is a small (14–20g) Australasian robin (Petroicidae) found in woodlands throughout Australia (Higgins and Peter, 2002). It is a “perch and pounce” predator, snatching tiny to small arthropods, either on the wing, from the ground surface or—less frequently—from vegetation (Recher and Davis, 1998). Although not a desert-adapted species, Jacky Winters rely entirely on dietary water, and surface water was unavailable to the study population during the data collection period. Pairs are territorial year-round (territory size is 1.7 hectares in southern Queensland; Wood et al., 2008) although they occasionally form cooperative groups of up to six birds (Higgins and Peter, 2002). Breeding occurs from September to January, with multiple clutches (comprised of two eggs) laid in open cup nests (Donaghey and Donaghey, 2017). At high temperatures, Jacky Winters use wing-spreading to facilitate



convective heat loss and panting for evaporative cooling (Sharpe et al., 2019, 2021).

Behavioral Data

Our color banded population of 40 breeding groups of Jacky Winters has been studied since May 2018. A subset of 16 groups have been habituated to the presence of an observer accompanying the birds at 3–5 m to allow accurate and unimpaired behavioral observations.

Behavioral data were collected from non-breeding birds between 8:00 and 16:00 during January and February 2020. Air temperatures during observation periods ranged from 15 to 43°C (Figure 1B; see “Weather and surface temperature data” for measurement details). We undertook 20-minute focal observation sessions, during which an observer and a scribe documented the following behaviors of the focal bird: all predator scares, social interactions (intra and intergroup squabbles and chases), non-foraging flights > 5 m and prey catching attempts (distance from perch to location of prey; location of prey: ground, air, leaves, trunk). Due to the very small size of the Jacky Winters' prey, we were unable to ascertain whether prey catching attempts were successful. We also documented the start and stop times of all bouts of preening, panting and wing-spreading (bouts were considered ended if the behavior ceased for > 10 s or was replaced by a different behavior). We recognized three wing-spreading postures: wing-raising (wings held slightly away from the sides of body), wing-drooping (wings held further away from body and wing-tips lowered beneath the level of the tail) and wind-surfing (wings held out horizontally, similar to flying position). We also noted changes to body posture and ptiloerection but did not quantify these due to the difficulty of defining them objectively. Additionally, every 2 min throughout the 20-min observation period, we recorded details of the focal individual's perch, estimating height above ground, degree of sun exposure (full sun, shade, dappled or overcast), direction faced (to nearest 45° compass point, using a *Garmin Etrex 30x* GPS), whether the bird was > 0.5 m from a living, naked (i.e., without dead fibrous bark) trunk/branch > 8 cm in diameter, and size of tree (diameter of the tree's largest trunk at 1.5 m). Prior to data collection, all observers were trained to estimate distances and heights accurately using a Bosch digital laser distance measure. During observation periods, we paused all data collection if the focal individual ceased to be visible and resumed when the bird could be seen again. If the focal bird could not be relocated within 10 min-, we aborted the observation session. Sessions of < 5 min were discarded.

We collected behavioral observations for 17 adult, non-breeding Jacky Winters (nine males, eight females) from 10 habituated groups. To ensure we obtained a balanced sample across all individuals, time of day and temperatures, we divided the 8-h data collection period into four 2-h blocks and classified days as either mild (<35°C), warm (35–38°C) or hot (>38°C) based on the maximum temperature for that day. To select which bird to sample, we chose individuals randomly from the pool of unsampled birds for that specific temperature/time of day category. Every individual was sampled during all four time periods and on cool, warm and hot days, with an average of

14.4 ± 0.8 observation sessions obtained for each bird (range 11–21). In total, we collected 79 h of behavioral data and 2,379 records of perch choice.

Weather and Surface Temperature Data

To assess how weather variables influenced the birds' behavior we used weather data collected every 30 min within 6 km of our study site by the Calperum-Chowilla OzFlux land-atmosphere observatory, operated by the Terrestrial Ecosystem Research Network (TERN). Windspeed and wind direction were measured 2 m above ground by a RM-Young Wind Sentry. At a height of 20 m, vapor pressure was measured by a LI-COR LI-7500 infra-red gas analyzer, and incoming shortwave radiation, by a large Kipp and Zonen CNR4. Air temperature in the shade was measured at a height of 2 m by a Vaisala HMP-45C temperature probe. We used this OzFlux weather data for all analyses of the birds' behavior. Because our behavioral observation sessions were shorter than the weather logging interval (20 versus 30 min), when allocating weather data to a session that occurred between weather readings, we selected the weather reading closest to the observation period (i.e., always within 10 min).

To obtain an indication of the thermal profile of the bird's habitat, we measured surface temperatures at 83 trees (all mallee eucalypts) within 10 Jacky Winter territories. All territories were within areas that had remained unburnt for at least 50 years. Temperatures were measured on clear afternoons during January 2020 (16:00 –19:30) when air temperature was ≥ 35°C (mean 39.5°C; range 35–47°C). We used a Digitech (model QM7221) infrared laser digital thermometer gun at a distance of 2cm, to measure all surface temperatures.

When choosing which trees to sample, we randomly selected up to five GPS waypoints from within each group's territory (waypoints represent a location where the territory-owners have been observed, and a pool of approximately 35 waypoints were available for each group). At the waypoint location, we measured air temperature at a height of 1.5 m using a Falcon Kestrel 2000 pocket wind and temperature meter (0.5°C accuracy) positioned within our own shade. We also took three ground surface temperature measurements (sun-exposed sand, shaded sand and shaded leaf litter), all sampled > 2 m from a tree trunk. We then selected two trees: the nearest to the waypoint and the largest (based on trunk circumference) within a 20 m radius of the waypoint. At each tree, we identified the thickest trunk (because mallee eucalypts are multi-stemmed) and measured the shaded surface temperature of this trunk at its base and at a height of 1.5 and 2.5 m. We also recorded its circumference at 1.5 m. We identified any potential refuges (crevices, spouts, hollows, etc.) at the tree's base and measured the surface temperature within these refuges at the deepest/shadiest location. We estimated the extent of leaf litter cover beneath the tree (the percentage of ground covered by leaf litter within a circle, 1 m in diameter, centered on the largest trunk) and measured the shaded surface temperature of this leaf litter.

Statistical Models

We constructed a series of linear and zero-inflated mixed models to explore relationships between behavior, habitat characteristics

and weather. Explanatory variables were chosen based on hypothesized drivers, including potential non-linear effects and interactions.

1. Foraging behavior. To test the hypothesis that foraging behavior will decline with increasing heat load (Youngentob et al., 2021), we constructed two separate models, with rate of prey capture attempts and distance flown to obtain prey as response variables. In each model we included the following fixed effects: air temperature and solar radiation (contributors to heat load; Mitchell et al., 2018), wind speed (because wind reduces heat load due to enhanced cooling; Wolf and Walsberg, 1996) and two-way interactions between these weather variables. Because microhabitat choice may impact foraging behavior (Cunningham et al., 2015), we also included perch height, perch height $\times$ air temperature and proportion of perches in shade as fixed effects. We included individual and group identity as random terms, to account for multiple sampling of the same birds and groups. We controlled for the following variables by including them as fixed effects: time of day (as the number of minutes after sunrise), the bird's sex (male or female) and observer identity (to account for any differences among observers). Although observers were trained in length estimation prior to data collection, we found that one observer slightly, but consistently, over-estimated while another slightly, but consistently, under-estimated. This resulted in a significant difference between observers in the quantification of parameters that involved estimating distance or trunk width. By including "observer" as a fixed effect, the model takes into account this source of variation so that it does not impact upon our findings relating to weather variables. In one model (Table 1B), we included observer as a random effect, rather than a fixed effect, because the model otherwise failed to converge. In this case, it was preferable to include observer as a random term to account for differences in the number of behavioral watches undertaken by each observer. In the model assessing distance flown to prey, we also included the location of the potential prey item (four categories) as a fixed effect. In a third model, we tested the hypothesis that birds will pre-emptively increase their foraging effort on the mornings of very hot days, using rate of prey catching attempts between 08.00 and 10.00 h as the response variable. We included the day's maximum air temperature as a categorical variable (>38 or $<38^{\circ}\text{C}$) and the same random terms as above.
2. Non-foraging behavior. To test the hypothesis that non-foraging motor activities will decline with increasing heat load (Cunningham et al., 2021), we constructed three separate models with the response variables: rate of non-foraging flights (>5 m), rate of social interactions and proportion of time spent preening. We included the three weather variables and their two-way interactions as fixed effects, controlled for sex and time of day, and used the same random terms as (1).
3. Heat dissipation behaviors. To assess the influence of weather variables on heat dissipation behaviors we ran two models with the following response variables: the proportion of time spent panting and the proportion of time spent wing-spreading (combining wing-raising, wing-drooping and wind-surfing into a single variable). In both models we included the same terms as in (2) plus a quadratic term for air temperature because we expected a non-linear response due to threshold-related responses. In the panting model we fitted vapor pressure, and its interaction with temperature, as additional fixed effects because humidity is known to affect panting behavior at high temperatures (Mitchell et al., 2018).
4. Perch choice. We hypothesized that the birds' choice of microsite will alter with increasing heat load, so we tested how the three weather variables were related to the following response variables: likelihood of perching in the sun, perch height, size of the perch tree, whether perched within 0.5 m of a naked branch/trunk (Briscoe et al., 2014) and whether facing the wind (the last two variables were categorical: yes/no). We tested each of these response variables in a separate model. We fitted the same fixed effects, including interactions and random effects, as in (2) and included air temperature as a quadratic term because we expected responses to be non-linear due to threshold effects.
5. Surface temperatures of mallee eucalypts. To test the hypothesis that the relative surface temperature of a tree's microsites is negatively related to tree size and leaf-litter ground cover, we ran five separate models with the following five response variables: surface temperature of leaf-litter at tree-base, tree-base refuges (crevices and hollows), tree trunk at its base, tree trunk at 1.5 m and tree trunk at 2.5 m. We fitted air temperature, tree size (trunk circumference at 1.5 m) and percentage leaf-litter cover as fixed effects. Models were fitted using the `lm` function because there were no repeat measures.

Model Fitting

For each model we fitted all potential explanatory variables (as described above), then removed non-significant terms sequentially, starting with interactions, to arrive at a final model with only significant or near-significant terms. The model with the lowest AIC score was chosen, which always only included significant or near-significant variables. To avoid multicollinearity among explanatory variables we first estimated pair-wise Pearson's correlation coefficients and confirmed that correlations were not high ($|r| < 0.7$). Model fit was checked using diagnostic plots to confirm model residuals were distributed around zero. For linear models we used residual plots and normality plots to check for deviations from normality among residuals, and for zero-inflated models we used DHARMa package (Hartig, 2020) to check for overdispersion.

Linear mixed models were fitted with REML (Restricted maximum likelihood) using the `lmer` function in the `lme4` package (Bates et al., 2015) and generalized linear mixed models (GLMM) were fitted using the `glmer` function in the `lme4` package

TABLE 1 | Results of REML mixed effects models testing for factors associated with foraging behavior.

Variables	Estimate	SE	t	p
A. Frequency of prey capture attempts				
Intercept	31.370	2.965	10.578	<0.001
Minutes after sunrise	−5.123	1.678	−3.054	0.003
Air temperature	−1.760	1.713	−1.028	0.305
Solar radiation	−1.842	1.823	−1.011	0.313
Perch height	−3.461	1.500	−2.307	0.022
Proportion of perches in shade	−2.669	1.602	−1.666	0.097
Air temperature × solar radiation	−4.806	1.561	−3.078	0.002
B. Frequency of prey capture attempts in morning (8–10 am).				
Intercept	35.11	3.61	9.727	0.000
Day's maximum temperature > 38°C	12.06	5.11	2.360	0.022
C. Distance flown to obtain prey				
Intercept	1.386	0.035	39.983	<0.001
Sex (male)	0.103	0.334	3.097	0.007
Air temperature	0.014	0.013	1.126	0.260
Windspeed	0.019	0.013	1.456	0.146
Solar radiation	0.017	0.014	1.240	0.215
Perch height	0.032	0.012	2.577	0.010
Location of prey (leaves)*	−0.421	0.039	−10.703	<0.001
Location of prey (trunk)*	−0.404	0.027	−14.697	<0.001
Location of prey (ground)*	−0.043	0.292	−1.492	0.136
Air temperature × solar radiation	0.026	0.013	1.936	0.053
Air temperature × windspeed	0.047	0.012	3.876	<0.001
Observer (1)	0.088	0.033	2.632	0.009
Observer (2)	−0.080	0.029	−2.735	0.006

*Relative to Location of prey (air). Final models are shown, following sequential removal of non-significant variables; significant variables in bold.

(Venables and Ripley, 2002). Zero-inflated models (used for heat dissipation models) were fitted using the glmmTMB package with beta-binomial distribution. All models were fitted in R 3.3.2 (R Development Core Team).

Other Statistical Analyses

To compare the surface temperatures of different microsites within mallee eucalypt trees (except for potential refuges), we used the full data set and conducted a Repeated One-way ANOVA and Tukey tests for all pairwise comparisons, using R 3.3.2. We also undertook a second Repeated ANOVA to compare the surface temperatures of potential refuges with other microsites, restricting the analysis to trees that contained refuges. To test whether the difference between surface temperatures and air temperature changed with air temperature, we used Pearson correlation coefficients to compare the variable “surface temperature minus air temperature” with air temperature. These tests, and all other non-modeling statistical tests were conducted in Microsoft Excel 2016 v2111. Means are presented $\pm$ 1 SE.

RESULTS

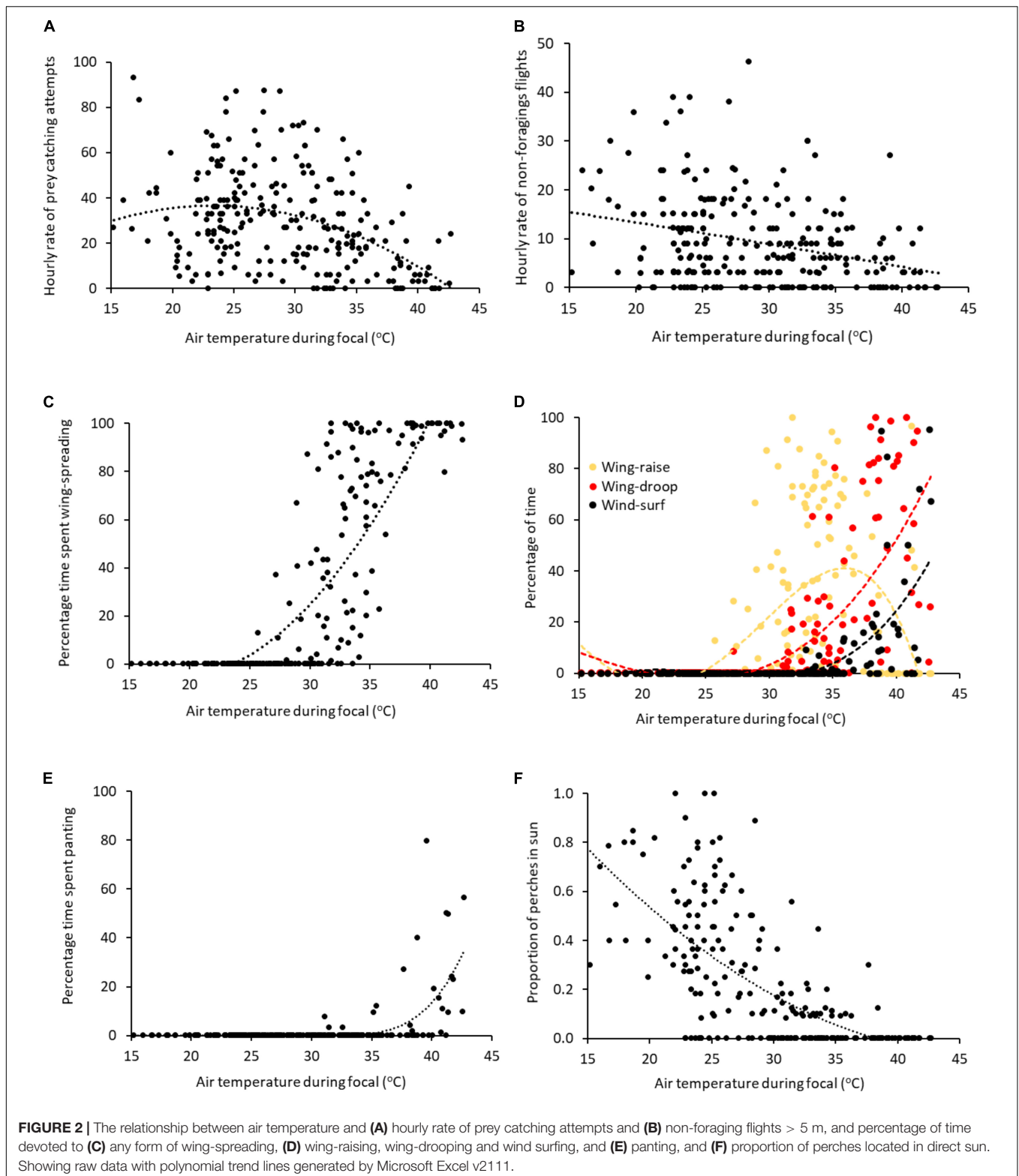
Foraging Behavior

Although there was no overall effect of either air temperature or solar radiation on foraging effort, when both these variables were

simultaneously high, Jacky Winters substantially reduced their foraging effort, after controlling for time of day (**Table 1A**: air temperature $\times$ solar radiation interaction; **Figure 2A**). At $> 35^\circ\text{C}$, the birds averaged only 12 (± 2) prey capture attempts per hour compared with 35 (± 2) at $< 28^\circ\text{C}$. Hourly rate of attempts was also negatively related to perch height and showed a negative trend with the proportion of a bird's perches that were in the shade (**Table 1A**).

To compensate for reduced foraging effort at high temperatures, individuals modified their time budget. On days $> 38^\circ\text{C}$, they increased their rate of foraging attempts in the mornings (8–10 am) by 30% and reduced afternoon foraging rates by 70%, (as compared with days $< 35^\circ\text{C}$) (**Table 1B** and **Figure 3**). To test if this increase in foraging effort was genuinely pre-emptive and that the birds were not simply compensating for a deficit of foraging on the previous afternoon, we focused on the hourly rate of foraging attempts at 8–10 am on days with a maximum temperature $> 38^\circ\text{C}$, comparing days that were preceded by a warm day ($35\text{--}38^\circ\text{C}$), when the birds foraged normally, and days preceded by a $> 38^\circ\text{C}$ day, when they abstained from afternoon foraging. There was no significant difference between the two (t -test: $t_{11} = 0.77$, $p = 0.46$; hourly attempts: warm: 50 ± 5 ; hot: 42 ± 9).

Jacky Winters did not modify the distance they flew to catch prey in response to air temperature, windspeed or level of solar radiation, although they flew further at high temperatures when



the wind was strong (Table 1C). The distance the birds flew to obtain prey was, however, positively correlated with perch height, but unrelated to whether the bird was perched in the shade. Males

flew further than females. Flight distance also varied with the prey's location, with birds flying furthest for aerial or ground prey but shorter distances for prey on leaves or tree trunks (Table 1C).

Other Activities

The hourly rate of non-foraging flights (>5 m) was negatively related to air temperature, after controlling for time of day (Figure 2B). This negative effect of increasing temperature on non-foraging flights tended to be exacerbated as solar radiation and windspeed increased (Table 2A: air temperature $\times$ solar radiation interaction, air temperature $\times$ windspeed interaction, respectively). Males moved more frequently than females (Table 2A). Hourly rates of social interactions were also negatively related to air temperature and to windspeed (Table 2B). However, the proportion of time spent preening showed a non-linear relationship with air temperature, increasing with temperature until the mid 30°Cs, then decreasing. Preening was also positively related to solar radiation, and this relationship was stronger as temperature increased (Table 2C: air temperature $\times$ solar radiation interaction).

Heat Dissipation Behaviors

The proportion of time spent wing-spreading (i.e., wing-raising, wing-drooping or wind-surfing) was positively associated with air temperature, although the relationship had a non-linear component (Table 3A and Figure 2C). The birds began wing-raising at air temperatures as low as 26°C and wing-drooping at about 32°C. Wing-raising was the most prevalent form of wing-spreading until around 35°C, when wing-drooping became more prevalent (Figure 2D). Proportion of time spent wing-spreading declined as windspeed increased, but the declining effect of windspeed on wing-spreading was reduced as air temperatures increased (Table 3A: air temperature $\times$ windspeed interaction).

Birds began panting at air temperatures in the 30°Cs, and proportion of time spent panting increased with air temperature (Figure 2E). Vapor pressure was also positively associated with time panting, but its effect on panting was less pronounced as air temperature increased (Table 3B: air temperature $\times$ windspeed interaction). Conversely, windspeed was negatively related to time spent panting: birds panted less when the wind was strong (Table 3B and Figure 4). Solar radiation was also negatively related to time panting.

Microsite Choice

The likelihood of a bird choosing to perch in direct sunlight was negatively correlated with both air temperature and level of solar radiation (Table 4A). In sunny conditions, birds began using shaded perches at air temperatures > 28°C. Only 1% of perches were in sun at temperatures > 35°C (compared with 47% at < 28°C; Figure 2F). Birds also shifted to shaded perches in windy conditions (Table 4A).

There was a non-linear relationship between the height of a bird's perch and air temperature, with the birds switching to elevated perches at temperatures > 35°C, when mean perch height almost doubled (from 2.7 to 5.1 m; Figure 5A and Table 4B). Perch height was negatively related to windspeed, with birds perching lower in high winds (Table 4B). Birds choosing high perches favored larger trees, with a positive correlation between perch height and perch tree size (Pearson's correlation: $r_{1978} = 0.34, p < 0.001$). The size of the perch tree was also related

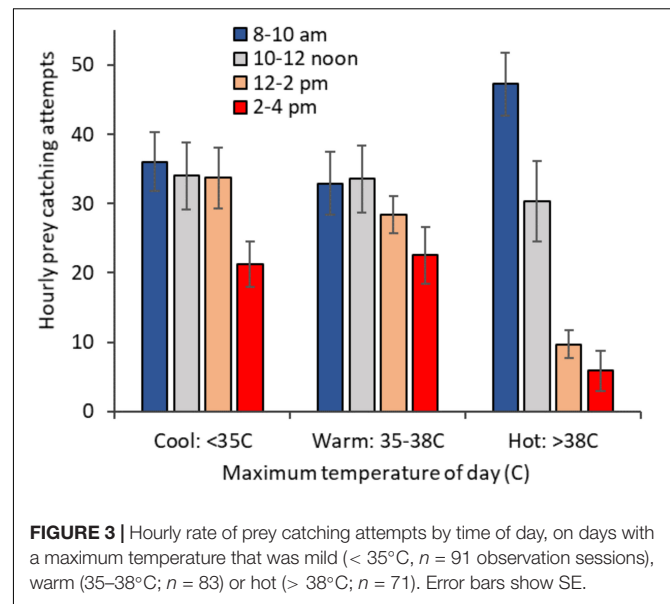


FIGURE 3 | Hourly rate of prey catching attempts by time of day, on days with a maximum temperature that was mild (< 35°C; $n = 91$ observation sessions), warm (35–38°C; $n = 83$) or hot (> 38°C; $n = 71$). Error bars show SE.

to air temperature, with birds favoring large trees at the highest temperatures (Figure 5B and Table 4C). Air temperature was also positively related to the likelihood of the birds perching within 0.5 m of a naked trunk or large branch (Table 4D).

Birds perched facing into the wind more often than expected by chance (44 versus 25%; Chi² goodness of fit: $X^2 = 434.38, n = 2,322, p < 0.001$) and this became more pronounced at high temperatures (67% at > 40°C; Table 4E). Facing into the wind was also positively related to windspeed, but negatively associated with level of solar radiation (Table 4E). Birds were also twice as likely to face into the wind if it was coming from the North (i.e., from the hot central Australian desert) as compared with other directions (78 versus 38%; Figure 6).

Once air temperature reached 39–40°C, Jacky Winters usually moved to the ground, sheltering in shaded crevices and depressions at the base of large trees. They did not perch on the ground under any other circumstances. At $\geq 39^\circ\text{C}$, 39% of our focal individuals retreated to a tree-base refuge, but this is a recognized underestimate because, once birds were within a refuge, we were unable to locate them to begin focal observations, so our sample was entirely biased to individuals that remained out of refuges. In total, we observed nine instances of Jacky Winters moving into tree-base refuges (eight individuals from four groups) but, at air temperatures $\geq 41^\circ\text{C}$, we observed only two individuals (from one group) remaining aloft.

Thermal Profile of Habitat

There was considerable thermal heterogeneity in the birds' habitat at air temperatures > 35°C (Figure 7). Sun exposure greatly increased surface temperatures, with sun-exposed sand 15.9 (± 1.2)°C hotter than air temperature and 7.3 (± 0.9)°C hotter than shaded sand, at an average air temperature of 39.5°C (Repeated one-way ANOVA

TABLE 2 | Results of REML mixed effects models testing for factors associated with non-foraging activities.

Variables	Estimate	SE	t	p
A. Frequency of non-foraging flights				
Intercept	-6.131	0.136	-45.21	<0.001
Minutes after sunrise	-0.240	0.072	-3.34	0.001
Sex (male)	0.226	0.110	2.06	0.039
Air temperature	-0.210	0.064	-3.28	0.001
Solar radiation	0.020	0.078	0.260	0.795
Windspeed	-0.130	0.070	-1.86	0.063
Air temperature × solar radiation	-0.139	0.075	-1.85	0.064
Air temperature × windspeed	-0.114	0.067	-1.71	0.088
Observer (1)	-0.516	0.168	-3.07	0.002
Observer (2)	0.267	0.135	1.97	0.048
B. Frequency of social interactions				
Intercept	-8.294	0.255	-32.58	<0.001
Air temperature	-0.414	0.137	-3.02	0.003
Windspeed	-0.435	0.161	-2.70	0.007
C. Proportion of time spent preening				
Intercept	-2.451	0.164	-14.911	<0.001
Minutes after sunrise	0.463	0.086	5.367	<0.001
Air temperature	5.299	1.080	4.905	<0.001
Air temperature²	-5.968	1.102	-5.414	<0.001
Solar radiation	0.309	0.117	2.646	0.008
Air temperature × solar radiation	0.364	0.121	3.009	0.003

Final models are shown, following sequential removal of non-significant variables; significant variables in bold.

TABLE 3 | Results of zero-inflated models with beta-binomial distribution (glmmTMB) testing for factors associated with the proportion of time spent undertaking heat dissipation behaviors.

Variables	Estimate	SE	Z	p
A. All wing-spreading behaviors				
Intercept	-6.503	0.509	-12.771	<0.001
Air temperature	26.129	3.062	8.535	<0.001
Air temperature²	-20.360	2.562	-7.947	<0.001
Windspeed	-0.890	0.251	-3.542	<0.001
Air temperature × windspeed	0.548	0.218	2.509	0.012
B. Panting				
Intercept	-8.317	0.748	-11.116	<0.001
Air temperature	3.225	0.435	7.414	<0.001
Solar radiation	-0.630	0.289	-2.181	0.029
Windspeed	-1.004	0.329	-3.055	0.002
Vapor pressure	1.465	0.551	2.659	0.008
Air temperature × vapor pressure	-0.919	0.302	-3.040	0.002

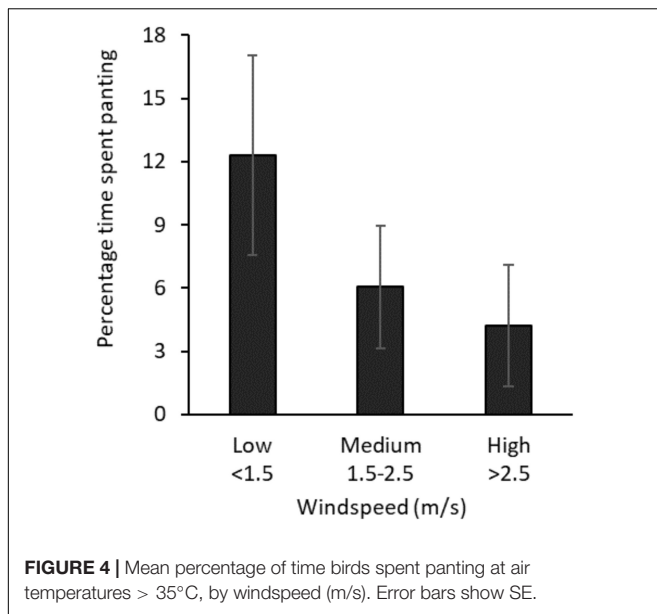
Final models are shown, following sequential removal of non-significant variables; significant variables in bold.

for surface temperatures, excluding refuges: $F_{6,81} = 189.1$, $p < 0.001$; Tukey: $p < 0.001$). The temperature difference between sun-exposed sand and air temperature increased as air temperature increased (Pearson's correlations: $r_{42} = 0.442$, $p = 0.003$).

Trees reduced ground surface temperatures, with shaded leaf-litter at the base of a tree $3.6 (\pm 0.4)^{\circ}\text{C}$ cooler than shaded leaf-litter > 2 m from a tree (Tukey: $p < 0.001$). The average temperature of shaded, tree-base litter was the same as air temperature at air temperatures $\leq 38^{\circ}\text{C}$ but rose to 6°C above air temperature at air temperatures $\geq 45^{\circ}\text{C}$ (Pearson's:

$r_{76} = 0.481$, $p < 0.0001$). The size of the tree (circumference of the tree's largest trunk) was negatively related to surface temperatures beneath the tree, with larger trees being cooler at all measured locations, except for the trunk at 2.5 m (Tables 5A–D and Figure 8).

Leaf-litter also moderated ground surface temperatures with shaded leaf-litter $1.4 (\pm 0.3)^{\circ}\text{C}$ cooler than shaded sand (both measured > 2 m from a tree, at an air temperature of 39.5°C ; Tukey: $p = 0.047$). The greater the proportion of ground covered by leaf-litter (within 1 m of the tree base), the cooler were the surface temperatures at the base of the tree (Tables 5A,B).



Tree-Base Refuges

We documented two types of potential thermal refuge at the base of mallee eucalypts: tree hollows and spouts (holes within a dead or living trunk < 50 cm from ground; $n = 20$; **Figure 9a**) and small crevices that provided partial shelter (nooks among the tree-base roots or depressions on the basal lignotuber; $n = 11$; **Figure 9b**). We included crevices in our sample because Jacky Winters used this refuge type most often.

The surface temperature within potential tree-base refuges (crevices and hollows) was significantly cooler than all other locations (Repeated one-way ANOVA: $F_{7,31} = 189.1$, $p < 0.001$; **Figure 7**). Tree-base hollows were $2.0 (\pm 0.8)^{\circ}\text{C}$ cooler than air temperature, but tree-base crevices did not differ significantly from air temperature (Paired t -tests: hollows: $t_{19} = 2.368$, $p = 0.029$; crevices: $t_{10} = -1.595$, $p = 0.142$). Tree size affected the thermal benefit of tree-base crevices and hollows, with bigger trees providing cooler refuges (**Table 5D** and **Figure 9c**).

Large trees were also more likely to contain tree-base refuges (**Figure 9d**): refuge-bearing trees were significantly larger (trunk circumference) than those without (t -test: $t_{25} = 3.016$, $p = 0.006$). Tree-base hollows did not occur in mallees with a trunk circumference < 30 cm (at a height of 1.5 m). Overall, refuges were rare: 63% of trees offered no potential refuges, even though we biased the sample toward large trees (by sampling the largest tree within 20 m at each sampling location).

DISCUSSION

Behavioral Responses to Heat

Jacky Winters exhibited a broad repertoire of thermoregulatory behaviors, which was nuanced, flexible and highly responsive to both air temperature and other abiotic factors, such as wind velocity and solar radiation. The sequence of their behavioral responses to heat is shown in **Figure 10**. At air

temperatures > 28°C, the birds withdrew to shade and reduced physically demanding activities such as non-foraging flights and social interactions. Air temperature *per se* did not reduce foraging activity (except at temperatures $\geq 38^{\circ}\text{C}$), suggesting that energy and water gains from feeding outweighed the costs of increased thermogenesis (Wolf and Walsberg, 1996). However, this balance shifted when air temperature and solar radiation were high simultaneously, and the birds curtailed foraging in response to this greater heat load. This reduction in foraging may have been influenced by heat-related changes to prey availability (Holm and Edney, 1973), but experimental studies show that birds given unlimited food still do not forage at high temperatures (digestion generates metabolic heat; Beale et al., 2018), suggesting that heat, not food availability, is responsible (Ricklefs and Hainsworth, 1968; Olinger, 2017).

The Jacky Winters also began adopting behaviors that facilitated convective heat loss at air temperatures in the high 20°Cs. In addition to wing-raising, to increase surface area to mass ratio, the birds flattened their plumage to reduce its insulative properties, and adopted a tall, upright posture to elongate body shape (**Figure 10**). As air temperature rose, wing-spreading became more exaggerated (becoming wing-drooping; **Figure 10**) and, when > 35°C, the birds moved to elevated perches in the largest trees. This preference for high perches appeared to be related to wind velocity, which increases with elevation (McIlveen, 1992). Average windspeed at our site doubled between the heights of 2 and 10 m. Choosing the windiest microsites allowed the birds to maximize “forced” convective cooling, and it is notable that they did not use elevated perches if the wind was already strong. The thermal benefit of higher wind velocity is evidenced by our finding that birds devoted less time to heat-dissipation behaviors (e.g., wing-spreading and panting) with increasing windspeed, which is consistent with laboratory findings (Wolf and Walsberg, 1996; Wolf et al., 2000). Jacky Winters were also three times more likely to perch facing into the wind at high air temperatures (>40 versus <20°C), probably to enhance convective heat loss, because narrow objects, pointing into the wind, lose heat more rapidly (Mitchell et al., 2018). Facing into the wind also facilitated wind-surfing (i.e., allowing the wind to lift the wings into flying position; **Figure 10**), enabling air flow over a greater surface area. By seeking out windy microsites and maximizing convective cooling, birds can reduce their heat load without relying on more costly evaporative cooling.

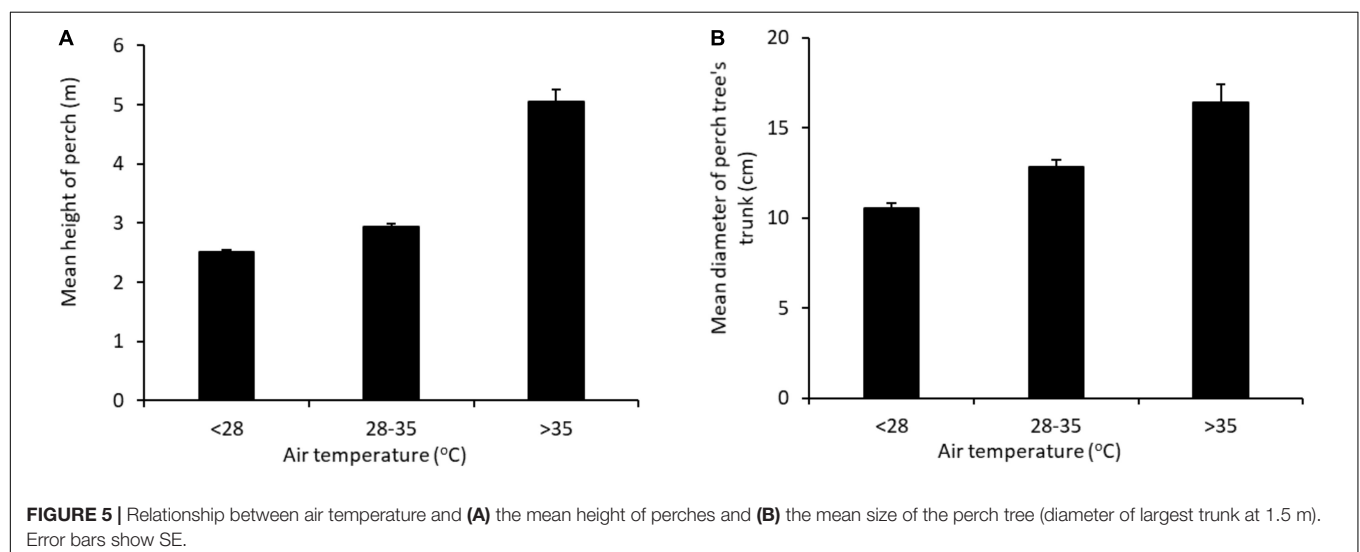
At air temperatures in the high 30°Cs, the birds also favored perches close to naked, living branches or trunks, and some individuals were seen lying with their bellies against the wood (a posture only adopted during extreme heat or incubation; Sharpe et al., 2021). This presumably facilitated conductive cooling because the surface temperature of living timber has been shown to be significantly cooler than air temperature, due to water flow in the tree’s phloem tissue and thermal inertia (Briscoe et al., 2014). The birds may also have favored perches close to large branches to capitalize on the dense shade cast by large limbs, because unbroken shade is rare in this habitat.

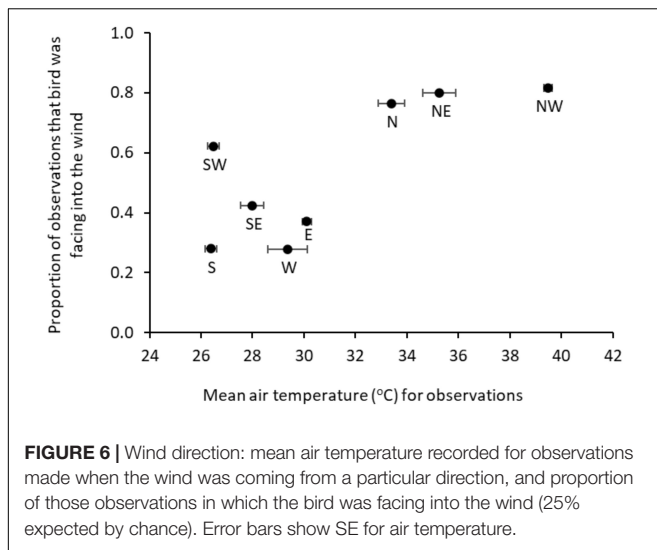
As air temperature approached body temperature (approximately 40°C; Boyles et al., 2011), the birds began

TABLE 4 | Results of REML or GLMM mixed effects models testing for factors associated with perch choice.

Variables	Estimate	SE	T	p
A. Likelihood of perching in sun				
Intercept	0.229	0.017	13.518	<0.001
Minutes after sunrise	−0.068	0.017	−3.985	<0.001
Air temperature	−0.123	0.015	−8.156	<0.001
Solar radiation	−0.049	0.019	−2.533	0.012
Windspeed	−0.066	0.015	−4.510	<0.001
B. Perch height				
Intercept	1.293	0.0454	28.484	<0.001
Air temperature	−0.415	0.1830	−2.270	0.024
Air temperature²	0.545	0.1836	2.970	0.003
Windspeed	−0.072	0.0191	−3.769	<0.001
C. Size of perch tree				
Intercept	2.488	0.1167	21.317	<0.001
Minutes after sunrise	0.056	0.0292	1.924	0.056
Air temperature	−1.809	0.2921	−6.193	<0.001
Air temperature²	2.003	0.2960	6.768	<0.001
Observer (1)	0.143	0.0841	1.702	0.090
Observer (2)	−0.3089	0.0772	−6.193	<0.001
D. Within 50cm of bare limb/trunk				
Intercept	−3.733	0.5319	−7.017	<0.001
Minutes after sunrise	0.581	0.2480	2.343	0.019
Air temperature	0.989	0.2624	3.768	<0.001
Observer (1)	1.617	0.5998	2.696	0.007
Observer (2)	−1.617	0.6126	−2.640	0.008
E. Facing into wind				
Intercept	−0.392	0.1160	−3.375	0.001
Minutes after sunrise	−0.249	0.1319	−1.891	0.059
Air temperature	0.524	0.1301	4.026	<0.001
Windspeed	0.523	0.1318	3.970	<0.001
Solar radiation	−0.418	0.1352	−3.088	0.002
Air temperature × solar radiation	−0.251	0.1247	−2.014	0.044

Final models are shown, following sequential removal of non-significant variables; significant variables in bold.





panting intermittently to facilitate evaporative cooling. Time spent panting increased with air temperature but was relatively low (averaging 13 min per hour at air temperatures $\geq 40^{\circ}\text{C}$) presumably reflecting the costs associated with this behavior. Panting uses body water, with rates of water loss increasing linearly with temperature (McKechnie et al., 2017). At air

temperatures of 40°C , a small bird loses about 3% of its body mass per hour (e.g. the Lesser Goldfinch, *Spinus psaltria*; Albright et al., 2017), so the risk of lethal dehydration during prolonged heat exposure is extreme for birds without access to water. Evaporative cooling is also less efficient in humid conditions (Mitchell et al., 2018) and Jacky Winters spent more time panting (an autonomic response to body temperature) when vapor pressure was high.

Once temperatures rose into the 40°C s, and dry (sensible) cooling was impossible, most of the birds retreated to the ground, taking refuge in shaded crevices or depressions at the base of large trees. These crevices were significantly cooler than any other location we measured (Figure 7). Inside refuges, the birds huddled together and lay with their bellies contacting the sand to facilitate conductive cooling. Jacky Winters do not normally perch within 5cm of conspecifics (Sharpe LL, pers. obs.), yet we observed one pair huddling with a territorial intruder they had been chasing earlier in the day. A similar breakdown of territoriality occurs in Arizona Grasshopper Sparrows (*Ammodramus savannarum ammodramus*) at high temperatures when the birds congregate in dense shrubs (Ruth et al., 2020). Huddling reduces the amount of surface area an animal exposes to the environment (Gilbert et al., 2010), which lessens heat gain when environmental temperature exceeds body temperature. Huddling inside a refuge also reduces evaporative water loss, due to increased humidity within the

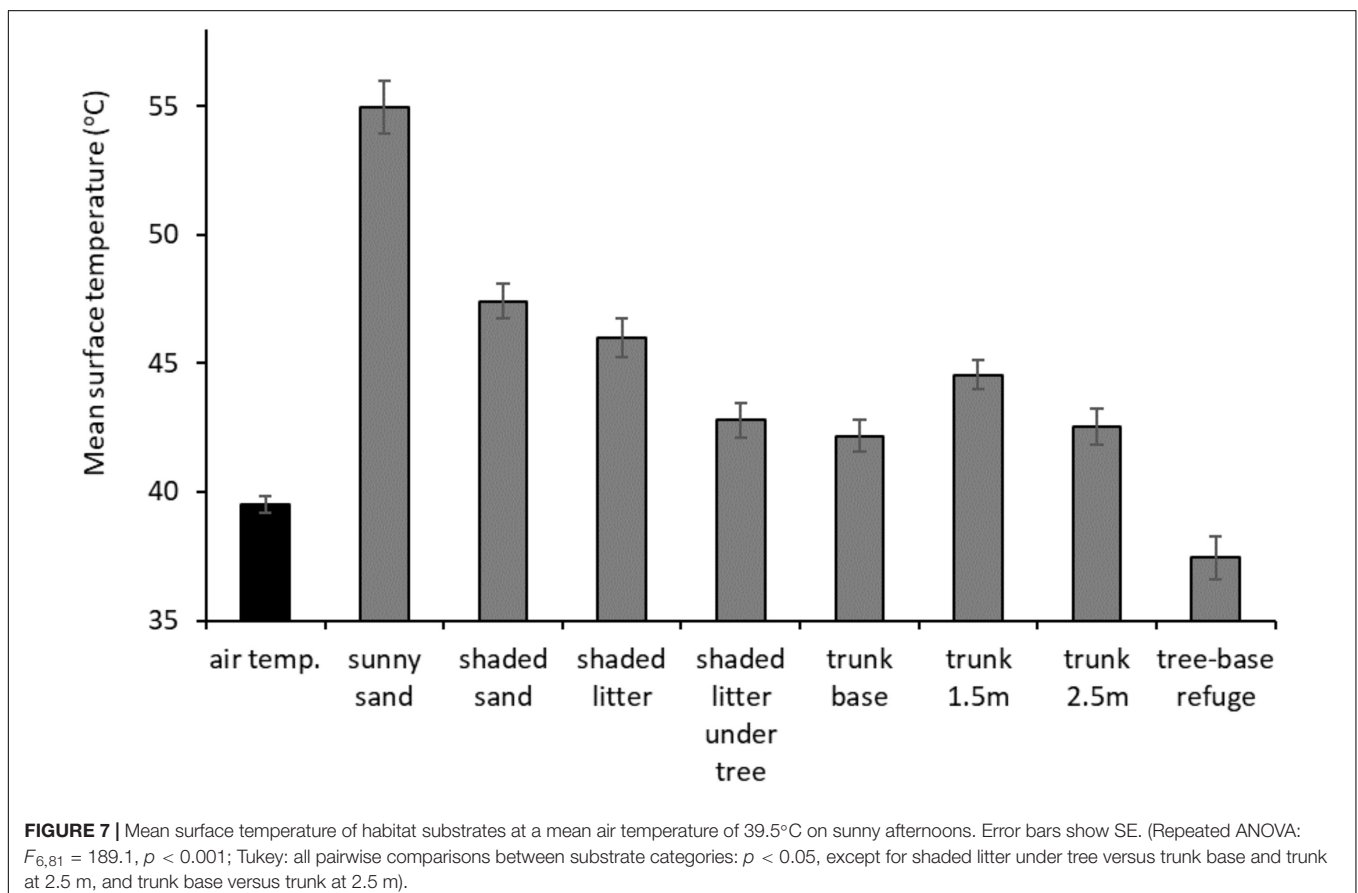
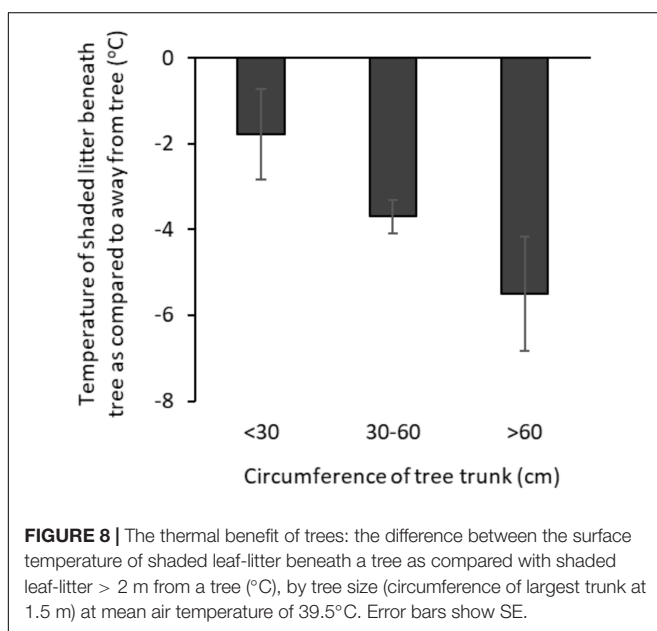


TABLE 5 | Results from linear models (lm) testing for factors associated with surface temperatures of mallee eucalypt trees, at air temperatures > 35°C.

Variables	Estimate	SE	t	p
A. Leaf litter at tree base				
Intercept	42.256	0.362	116.588	<0.001
Air temperature	5.057	0.374	13.536	<0.001
Tree size (trunk circumference)	−1.331	0.427	−3.116	0.003
Leaf litter coverage	−0.952	0.364	−2.616	0.011
B. Base of tree trunk				
Intercept	42.184	0.326	129.617	<0.001
Air temperature	4.460	0.331	13.483	<0.001
Tree size (trunk circumference)	−1.144	0.328	−3.488	<0.001
Leaf litter coverage	−0.790	0.329	−2.402	0.019
C. Potential tree base refuge (crevices)				
Intercept	39.192	0.862	45.448	<0.001
Air temperature	2.737	1.035	2.645	0.014
Tree size (trunk circumference)	−1.306	0.611	−2.138	0.042
Leaf litter coverage	−1.289	0.698	−1.847	0.762
D. Tree trunk at 1.5 m				
Intercept	44.558	0.277	160.607	<0.001
Air temperature	4.534	0.282	16.078	<0.001
Tree size (trunk circumference)	−0.761	0.280	−2.719	0.008
Leaf litter coverage	−0.373	0.280	−1.329	0.188
E. Tree trunk at 2.5 m				
Intercept	42.556	0.402	105.753	<0.001
Air temperature	5.130	0.407	12.619	<0.001
Tree size (trunk circumference)	−0.760	0.405	−1.876	0.065
Leaf litter coverage	−0.280	0.406	−0.689	0.493

Full models are presented; significant variables in bold.

refuge (Luna et al., 2020), which is potentially critical for survival during prolonged heat exposure. The thermal and water-saving benefits of huddling may explain why, on the hottest days, we



repeatedly observed large, mixed-species aggregations of small passerines sheltering together within single tree-base refuges.

Most Jacky Winters modified their behavior and microsite use to minimize convective heat transfer once air temperatures rose above body temperature. This behavioral transition, when heat gradients reverse, may prove to be critical for endotherms now experiencing unprecedented heat. Although we expect organisms to adopt behaviors that maximize their fitness, this is not always the case when individuals encounter novel conditions (e.g., responses to novel predators, artificial light, anthropogenic food; Tuomainen and Candolin, 2011). Although some species may have sufficient cognitive and behavioral flexibility to cope with this transition point (Ghalambor et al., 2007; Mills and McGraw, 2021), for others, air temperatures above body temperature may act as an insurmountable barrier to effective behavioral thermal regulation. Australian flying foxes (*Pteropus* spp.), for example, exhibit a suite of thermoregulatory behaviors (e.g., wing-fanning to maximize convective and radiant cooling, shade-use, panting and saliva-spreading; Welbergen et al., 2008) but in extreme heat they do not seek cooler microsites or adopt behaviors that minimize dry heat exchange. As a consequence, they succumb to lethal heat stress at air temperatures > 42°C, and 45,500 (in 600 colonies in SE Queensland) died on a single day in 2014 (Welbergen et al., 2014). This vulnerability to extreme heat may be much more widespread than we recognize, particularly among small endotherms (McKechie et al., 2021) because few species

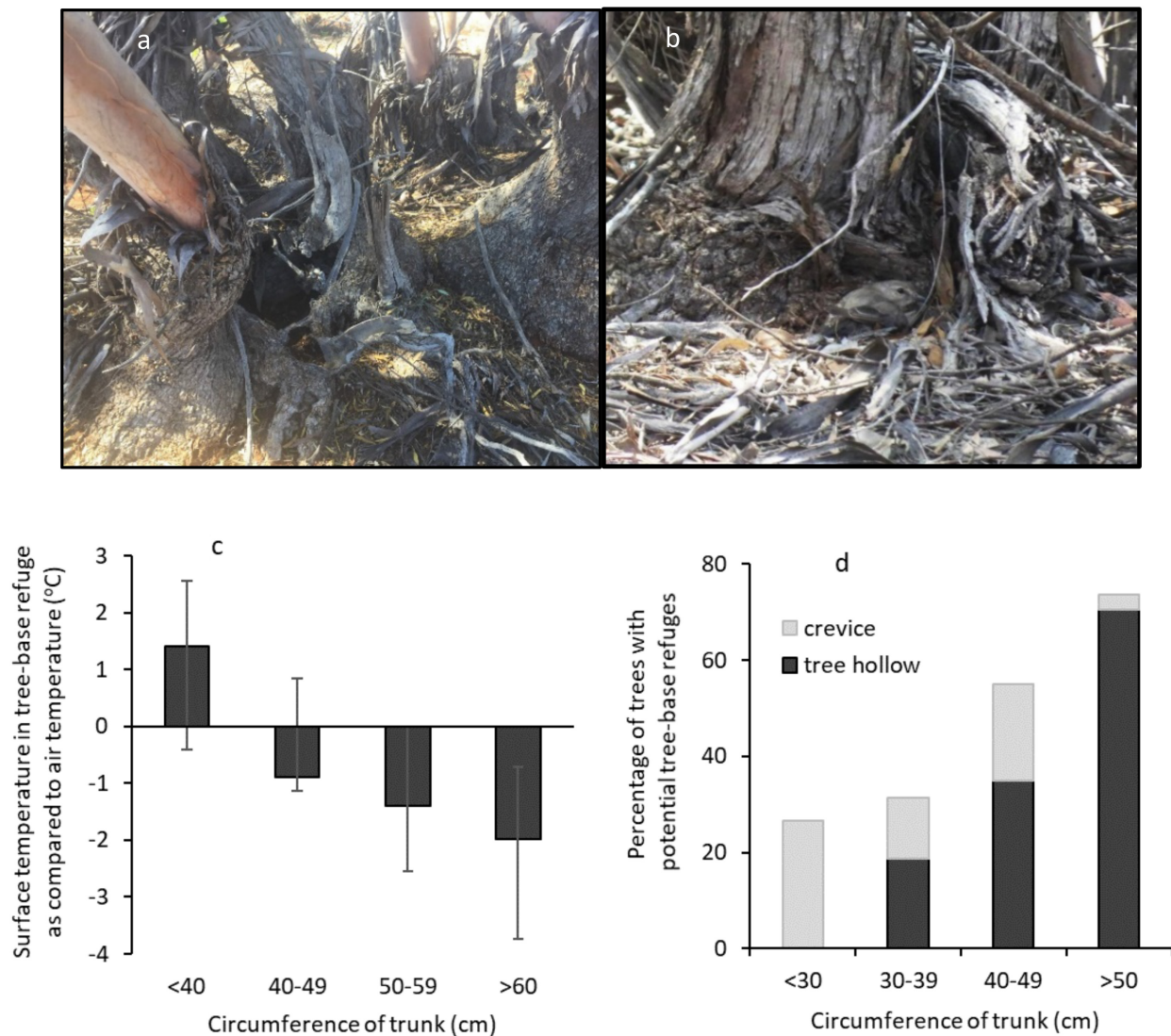


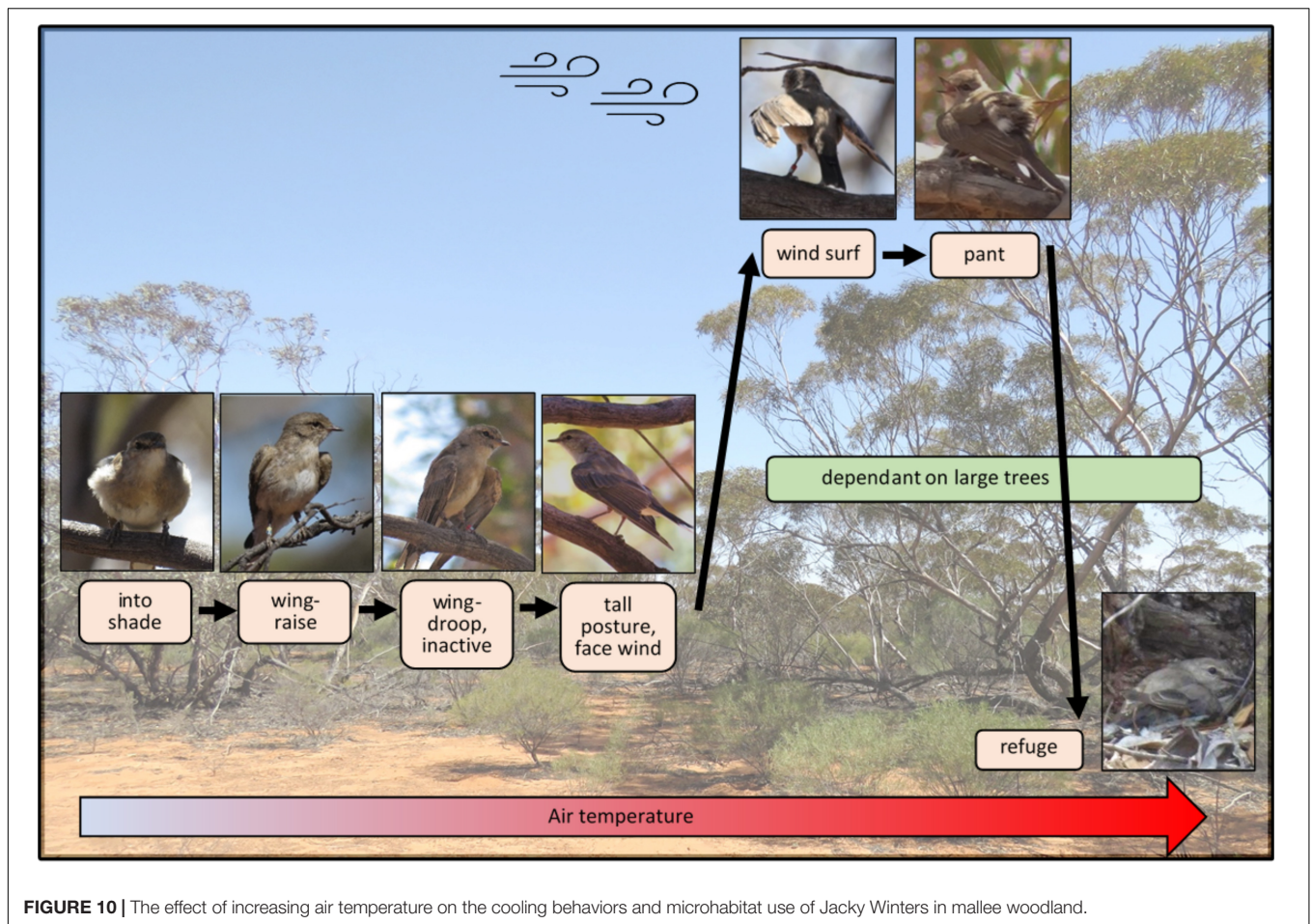
FIGURE 9 | Tree-base refuges: **(a)** a tree hollow and **(b)** a Jacky Winter sheltering in a crevice/depression. **(c)** Mean difference between the surface temperature in a tree-base refuge and air temperature (at a mean air temperature of 39.5°C) and **(d)** the percentage of mallee eucalypts with potential tree-base refuges, by tree size. Error bars show SE.

are as readily observable as flying foxes, which roost in large, noisy colonies, and attempts to quantify individual responses to extreme heat are rare (Buchholz et al., 2019).

Potential Costs of Behavioral Thermoregulation

Even when lethal stress is avoided, thermoregulatory behaviors carry opportunity costs, with organisms known to reduce sociality, territorial defense, food intake or offspring care (Cunningham et al., 2021). Panting, for example, reduces foraging success in birds that excavate subterranean prey with their beak (du Plessis et al., 2012; van de Ven et al., 2019) and the utilization of cooler microhabitats is often associated with a reduction in forage quality or foraging success

(Mason et al., 2017; van de Ven et al., 2019). With greater heat exposure, these costs may become unsustainable. For example, in Mexico's *Sceloporus* lizards, the growing need for extended periods of inactivity during the breeding season, to avoid lethal heat stress, has resulted in the extinction of 12% of monitored populations (Sinervo et al., 2010). We found that the Jacky Winters' use of elevated perches carried foraging costs, (after taking into account the effect of temperature), with birds making fewer prey catching attempts and flying further to capture prey. Perching in shade may also impair the birds' ability to detect prey because they tended to make fewer foraging attempts from shaded perches. Southern Fiscals (*Lanius collaris*)—an African “perch and pounce” avian predator—consume 50% less prey when hunting from shaded perches (Cunningham et al., 2015), although



this could be due to decreased foraging effort associated with temperature or time of day.

Reduced food intake—due to the necessity for inactivity and voluntary fasting at high temperatures—is a major cost of thermoregulatory behaviors in endotherms (Youngentob et al., 2021). By mounting video cameras at refuges, we found that Jacky Winters remained within refuges for up to 8 h and only emerged just before sunset (when air temperature dropped to about 38°C), precluding any afternoon foraging. Under these conditions, the birds lose > 2% of body mass per day (Sharpe et al., 2019), and similar losses occur in other wild avian species (du Plessis et al., 2012; Gardner et al., 2016; van de Ven et al., 2019). Such loss of body condition is likely to have long-term consequences for individual health, fitness and survival (Cunningham et al., 2021; Youngentob et al., 2021). Although Jacky Winters can partially recoup lost body condition during the interval between heat waves (Sharpe et al., 2019), the predicted increase in the frequency and duration of heat wave events is likely to have a detrimental impact.

In an effort to compensate for lost foraging time on very hot days, the Jacky Winters appeared to pre-emptively increase their foraging effort on the mornings of extremely hot days (Figure 3). Given that this species is reliant solely on dietary water, obtaining sufficient food prior to prolonged heat exposure will be critical

for avoiding lethal dehydration. Pre-emptive foraging has also been observed in other avian species (Carroll et al., 2015; Cooper et al., 2019) but is reliant upon the birds' ability to anticipate forthcoming conditions. Overnight temperature seems to be the most likely cue, as evidenced by an avian mass mortality event that occurred on a single, isolated hot day in Western Australia in 2010, when early morning temperatures were indistinguishable from those of previous, much cooler days (Cooper et al., 2019). The short-term predictability of heat waves may be critical to the survival of arid zone birds.

Thermally Buffered Microhabitats

The Jacky Winters' use of different microhabitats altered markedly as temperatures increased and at high temperatures the birds used the largest trees available to them. Surface temperatures at different microsites varied by up to a 35°C on the hottest days (air: > 44°C; sun-exposed sand: 77°C; tree-base refuge: 42°C) but thermally buffered microsites were rare. Tree-base refuges, the coolest sites, were only, on average, 2°C cooler than air temperature. The presence of trees and leaf-litter ground cover reduced surface temperatures, but tree size had the greatest influence: the larger the mallee eucalypt, the cooler the surface temperatures at its base, including within refuges (Figures 8, 9), presumably due to their larger, multitiered

canopies (Lindenmayer et al., 2014). This greater leaf area not only increases shade below the tree but amplifies levels of evapotranspiration (Sanusi et al., 2017). Transpiration from leaves directly reduces air temperature below a tree's canopy by 1 to 8°C, depending on species and environmental conditions (Rahman et al., 2020).

Large, fully mature mallee eucalypts also provided the birds with the opportunity to perch higher (giving them access to higher wind velocities) and were significantly more likely to contain potential tree-base refuges. In fact, tree-base hollows, the best quality refuges, were found only in the largest mallees (trunk circumference > 30 cm). The importance of large trees to the survival of Jacky Winters became apparent when air temperatures rose into the high 40°Cs. Although only one individual succumbed to acute heat stress/dehydration during the data collection period, 29% of our habituated adults died during a severe heat wave 3 weeks prior to data collection, when air temperature attained a record-breaking 48.8°C. These deaths appeared to be concentrated in parts of our study area that did not contain large trees, and although we did not verify this quantitatively, it underscores the importance of thermally buffered refuges for organisms facing unprecedented heat exposure.

Large, old trees act as keystone structures in many ecosystems, providing cavity nest-sites and unique microhabitats, facilitating habitat regeneration, storing large quantities of carbon and modifying ecosystem hydrology and water table level (Lindenmayer et al., 2014). However, the potential thermal benefits provided by old, large trees have yet to be quantified and we could find only one published study: in Canada's boreal forests, stands of mature conifers were significantly cooler than stands of younger trees or shrubland when ambient temperatures were highest, and grizzly bears (*Ursus arctos horribilis*) favored these old stands at high temperatures (Pigeon et al., 2017). Interspecific comparisons of urban trees reveal that size *per se* is not a determinant of a species' thermal buffering properties because small tree species often have denser canopies, and it is leaf area—and rate of evapotranspiration—that dictate cooling effect (Sanusi et al., 2017; Rahman et al., 2020). Arid adapted species provide less thermal benefit because they have lower transpiration rates. Although the thermal buffering provided by mallee eucalypts at our semi-arid site would be constrained by their restricted evapotranspiration rates and limited shade generation (the leaves of arid-adapted eucalypts are vertically aligned to minimize sun exposure at the hottest time of day; King, 1997), the largest trees at our site may have accessed ground water unavailable to smaller conspecifics (Lindenmayer et al., 2014), potentially augmenting their value as thermal refuges.

Climate Change and Adaptation Management

Our finding that the availability and value of thermal refuges increases with the size of mallee eucalypts has important management implications. Mallee stems are usually killed by wildfire and high intensity planned burns, hence trunk size

and tree height increase with time since fire (Yates et al., 2017; Clarke et al., 2021). Indeed, it takes 60 years for mallees to attain full canopy height, and 40–60 years to develop hollows in living timber (Haslem et al., 2011; Clarke et al., 2021), likely reflecting their value as thermal refuges. Climate change is expected to influence the incidence of wildfires in mallee habitat; in mallee regions of Victoria, for example, the number of days of extreme fire danger and the occurrence of dry lightning are predicted to increase (Clarke et al., 2021). Whether this leads to increased fire incidence depends on their interaction with reductions in fuel biomass and continuity, associated with increasing aridity (Bradstock, 2010). Nevertheless, any increase in fire frequency would prevent mallee eucalypts from attaining the large size required for thermal buffering.

The world's dryland areas are predicted to become increasingly arid, with worsening droughts (IPCC, 2021), which reduce vegetation cover and can permanently alter habitat structure (Pritzkow et al., 2021). At our study site, densely leaved, green shrubs—identified as critical thermal refuges in other terrestrial habitats (Carroll et al., 2015; Anthony et al., 2020; Ruth et al., 2020)—were simply not present during the data collection period due to 2 years of severe drought. In previous summers, we had observed many small passerines (although not Jacky Winters) sheltering within such shrubs at high temperatures. The loss of this microhabitat, combined with the reduction in leaf area that eucalypts suffer during drought (Pritzkow et al., 2021), is likely to seriously impair the ability of avian species to survive heat wave events.

Options to help biodiversity adapt to climate change are limited, but the relationship between fire interval, tree size and thermal refuges offers an opportunity for “low regrets” intervention (Prober et al., 2019) in mallee ecosystems. Managing fire to maintain and increase the area of mallee in older age classes is likely to maximize the availability of thermal refuges, thereby reducing mortality during heat waves. Later seral stages in mallee also provide significant habitat structural elements that support a diversity of reptiles, birds and mammals (Yates et al., 2017; Clarke et al., 2021). Another option worth considering relates to habitat structures known to act as critical refuges in other hot, arid ecosystems. Underground burrows are exploited by a huge array of species during extreme heat: four lark species use lizard burrows in the Arabian Desert (Williams et al., 1999) and more than 50 vertebrate species shelter in gopher tortoise (*Gopherus polyphemus*) burrows in south-eastern United States (Walde et al., 2009). However, burrows are no longer present in SE Australia's mallee woodlands due to the extirpation of at least three species of medium-sized, burrowing marsupial (Fleming et al., 2014). The reintroduction of burrowing species could greatly enhance the availability of thermally buffered microsites within this habitat.

Conclusion

Jacky Winters exhibited a broad and nuanced repertoire of thermoregulatory behaviors, which appeared “appropriate” to environmental conditions, based on principles of heat

transfer. However, the effectiveness of their behavioral strategies was dependent on the quality and availability of specific microhabitats. There is an urgent need for studies that quantify how wild individuals respond behaviorally to heat waves and identify which fine-scale components of their habitat serve as thermal refuges (Buchholz et al., 2019). Only once we have this information can we hope to develop effective conservation strategies to help to mitigate the adverse impact of rising global temperatures on biodiversity.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethics approval for this research was granted by the Australian National University Animal Experimentation Ethics Committee (A2018/29), and the work was approved by the South Australian Department of Environment and Water (E26755-1) and the Australian Bird and Bat Banding Scheme (153506).

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AUTHOR CONTRIBUTIONS

JG conceived the study and performed the statistical analyses. LS designed the methodology and wrote the manuscript. LS and JG collected the data. JG and SP reviewed the manuscript. All authors secured funding and approved the final manuscript.

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Extreme Hot Weather Has Stronger Impacts on Avian Reproduction in Forests Than in Cities

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Climate change and urbanisation are among the most salient human-induced changes affecting Earth's biota. Extreme weather events can have high biological impacts and are becoming more frequent recently. In cities, the urban heat island can amplify the intensity and frequency of hot weather events. However, the joint effects of heat events and urban microclimate on wildlife are unclear, as urban populations may either suffer more from increased heat stress or may adapt to tolerate warmer temperatures. Here, we test whether the effects of hot weather on reproductive success of great tits (*Parus major*) are exacerbated or dampened in urban environments compared to forest habitats. By studying 760 broods from two urban and two forest populations over 6 years, we show that 14–16 days-old nestlings have smaller body mass and tarsus length, and suffer increased mortality when they experience a higher number of hot days during the nestling period. The negative effects of hot weather on body mass and survival are significantly stronger in forests than in urban areas, where these effects are dampened or even reversed. These results suggest that urban nestlings are less vulnerable to extreme hot weather conditions than their non-urban conspecifics. This difference might be the result of adaptations that facilitate heat dissipation, including smaller body size, altered plumage and reduced brood size. Alternatively or additionally, parental provisioning and food availability may be less affected by heat in urban areas. Our findings suggest that adaptation to heat stress may help birds cope with the joint challenges of climate change and urbanisation.

Keywords: climate change, heatwave, urban heat island (UHI), offspring size, offspring mortality, thermal tolerance

INTRODUCTION

Current large-scale environmental changes are substantially influencing the ecological conditions for Earth's biota. Climate change is one of the dominant global processes that affect a wide range of organisms. Its effects include systematic changes in the long-term average meteorological conditions and also increases in the frequency of extreme weather events like heatwaves, droughts,

and heavy storms (Buckley and Huey, 2016; Drumond et al., 2020; Perkins-Kirkpatrick and Lewis, 2020). These climatic changes are occurring in a world that is experiencing global-scale transformation of natural habitats into heavily modified anthropogenic habitats. For example, current estimates show that 77% of the terrestrial habitats have already been modified by the direct effects of human activities such as agriculture and urbanisation (Watson et al., 2018), and the remaining pristine habitats are also disappearing at a high rate (Watson et al., 2016, 2018; Di Marco et al., 2019) forcing an increasing number of wildlife populations to persist in anthropogenic environments (Alberti and Marzluff, 2004). Although the ecological and evolutionary consequences of different global processes such as climate change and anthropogenic land-use conversion are most often investigated separately, they obviously do not act independently. Thus to better understand their current effects and to predict the changes likely induced in the future, we need to investigate their joint impacts in natural systems.

Heat events, including hot days and heatwaves, are among those extreme meteorological events that have become more frequent in the last few decades (Drumond et al., 2020). Extreme hot conditions can have strong biological impacts, affecting both survival and reproduction of organisms (Mora et al., 2017; Woodroffe et al., 2017; Conradie et al., 2019). For example, when hot days occurred for several consecutive days in Southern and Western Europe in 2003, it resulted in an estimated 70,000 heat-related human deaths (Robine et al., 2008) and was followed by detectable decreases in wild bird populations (Jiguet et al., 2006). However, even a single hot day can cause acute heat stress and have metabolic costs (Moagi et al., 2021). Extreme hot weather affects reproductive success negatively in a wide range of taxa, including both ectothermic (Zizzari and Ellers, 2011; Dayananda et al., 2017) and endothermic animals (Welbergen et al., 2008; Conrey et al., 2016; Marrot et al., 2017; Woodroffe et al., 2017; Van de Ven et al., 2020; Bourne et al., 2021; McCowan and Griffith, 2021; Oswald et al., 2021). These effects of extreme hot weather may be mediated by several, non-exclusive mechanisms, including both direct effects of heat stress on physiological and cognitive functioning (Welbergen et al., 2008; Danner et al., 2021; McCowan and Griffith, 2021) and indirect effects of heat *via* altered ecological conditions like food or water availability (du Plessis et al., 2012; Fuller et al., 2016).

Anthropogenic habitat change, especially urbanisation, is likely to interact with extreme weather events because it fundamentally alters several basic environmental conditions, including microclimate and food availability (Alberti and Marzluff, 2004; Seress and Liker, 2015). Urban heat island (UHI) refers to the generally higher ambient temperature in cities compared to the surrounding non-urban areas, which is largely generated by heat storage in buildings and sealed roads. Its intensity can be as high as +5°C in some metropolitan areas (Landsberg, 1981; Zhao et al., 2014; Central and Climate, 2021), so organisms in cities experience higher temperatures and more frequent heat events compared to organisms in non-urban areas. The UHI effect may interact with heat events in at least two ways. First, UHI can exacerbate the biological effects of extreme heat events. For example, models show that daytime temperature

in temperate-zone cities during a heat event can be higher by, on average, 2.8°C as a result of the synergistic effect between UHI and heat events (Zhao et al., 2018). Indeed, heat stress in cities can be higher during a heat event than the sum of the background UHI effect and the heat-event effect (Li and Bou-Zeid, 2013). According to these model predictions, the number of hot days is higher in urban than in non-urban areas with an additional increase in UHI resulting in increased human mortality (Li and Bou-Zeid, 2013; Rizvi et al., 2019). Similarly, animals are more likely to reach their upper limit of thermal tolerance in cities during heat events than in non-urban areas (Krause et al., 2017). Thus, urban populations may suffer a stronger reduction in survival and reproductive success than non-urban populations due to hot days (“superimposed heat and UHI effects” hypothesis).

Second, it is also possible that during long-term exposure to UHI, urban populations become adapted to higher temperatures (“adaptation to UHI” hypothesis). For example, heat tolerance (expressed as the thermal maximum where animals become unable to coordinate their motor performance) is higher in urban compared to rural populations in various ectotherm species (Brans et al., 2017; Diamond et al., 2017; Campbell-Staton et al., 2020), and these local thermal adaptations can have consequences for their reproductive success (Meineke et al., 2013; Diamond et al., 2017; Martin et al., 2021). However, endotherm species have different constraints and mechanisms for thermoregulation compared to ectotherms, as endotherms employ not only behavioural but also a variety of physiological mechanisms to regulate body temperature which have significant energetic and hydric costs (Levesque and Marshall, 2021). Although there is much less data on urban thermal adaptations in endotherms, the smaller body size of the rodent *Peromyscus maniculatus* (Guralnick et al., 2020) and reduced number of feathers in some birds (Sándor et al., 2021) in urban populations might also reflect adaptations to the UHI.

So far, only a few studies have tested the above hypotheses for joint effects of extreme hot weather events and habitat urbanisation. Their results show either additional mortality in cities in humans (Li and Bou-Zeid, 2013) or adaptation to UHI effects in water fleas (Brans et al., 2017). Importantly, there is no field study for endotherm animal populations, despite that these are the most frequent targets of conservation efforts and play crucial roles in urban ecosystems. To help fill this knowledge gap, we tested whether heat events influence avian breeding success differently in cities and forest habitats. We analysed 6 years of breeding biology data of two urban and two forest populations of great tits (*Parus major*), a small songbird that frequently breeds in both urban and forest habitats. In this species, similarly to several other birds, reproductive performance is typically lower in urban sites than in more natural areas (Bailly et al., 2016; Seress et al., 2018). We tested whether nestlings’ body mass, tarsus length, and survival are related to the number of hot days differently in urban and forest sites. According to the “superimposed heat and UHI effects” hypothesis, we expect that heat events would result in stronger negative impacts on reproduction in urban than

forest habitats. Alternatively, the “adaptation to UHI” hypothesis predicts that heat events would have reduced impact in urban than forest populations.

MATERIALS AND METHODS

Data Collection

We installed nest-boxes for great tits in an urban site (city of Veszprém 47°05′17″N, 17°54′29″E), and in two sites in natural habitats (forests near Vilma-pusztá 47°05′06.7″N, 17°51′51.4″E and Szentgál 47°06′39″N, 17°41′17″E) in 2012, and additionally in another urban site (Balatonfüred 46°57′30″N, 17°53′34″E) in 2013. Urban nest-boxes are located mostly in public parks, university campuses, and a cemetery, where vegetation contains both native and introduced plant species. Forest study sites are located in deciduous woodlands, characterized by beech *Fagus sylvatica* and hornbeam *Carpinus betulus* (in Szentgál) or downy oak *Quercus cerris* and South European flowering ash *Fraxinus ornus* (in Vilma-pusztá). See detailed description of study sites in Seress et al. (2018). In March 2013, we installed a WH 2080 weather station (Ambient, LLC, AZ, United States) at each study site that recorded hourly temperature (°C) data throughout the 6 years of the study.

Great tits usually raise one or two broods per breeding season. We collected data on all breeding attempts at each site from 2013 to 2018, by recording the number of eggs and nestlings in the nest-boxes every 3–4 days from March to the end of July. There were 48 to 108 nest-boxes available in each study site, and 12 to 77% of these nest-boxes was occupied by great tits during the study period (depending on the site and year). We captured parent birds using a nest-box trap 6–15 days after their first nestling had hatched. We determined parents' sex based on their plumage characteristics and banded each bird with a unique combination of a numbered metal band and three plastic color bands. Breeding adult birds that had been color-banded on previous occasions were identified from recordings made during the nestling period by using a small, concealed video camera attached to the nest-boxes (Seress et al., 2017). In these video samples, we considered a color-banded individual to be a parent bird if it was recorded to enter the nest-box with food at least once. Close to fledging (at day 14–16 post-hatch; day 1 being the hatching day of the first nestling in the brood), we measured nestlings' body mass with a Pesola spring balance (± 0.1 g) and tarsus length with a Vernier caliper (± 0.1 mm).

All procedures applied during our study were in accordance with the guidelines for animal handling outlined by ASAB/ABS¹ and Hungarian laws. We have all the required permissions for capturing, measuring of the birds and monitoring their breeding from the Government Office of Veszprém County, Nature Conservation Division (former Middle Transdanubian Inspectorate for Environmental Protection, Natural Protection and Water Management; permission number: 24861/2014 and VE-09Z/03454-8/2018) and from the National Scientific Ethical

Committee on Animal Experimentation (permission number: VE/21/00480-9/2019).

Data Processing

We used both the first and second annual broods of each pair in the study. We omitted broods where we could not precisely identify the nestling period due to unknown dates of hatching and/or the failure of the entire brood before hatching, and also those broods where we had gaps in the temperature data for more than 2 days during the nestling period ($n = 190$ broods). To characterize breeding success in each brood, we focused on the size and survival of nestlings. Offspring mass is a good indicator of post-fledging survival in birds (Ronget et al., 2018), so we preferred this variable over indices of “body condition” that express body mass in relation to length, because the latter indices can be inadequate for quantifying “fatness” in growing nestlings (Kraft et al., 2019).

For analysing the effects of weather on nestling size, we used the average body mass and the average tarsus length of each brood as response variables, and we included broods where at least one offspring was alive at the time of nestlings' measuring and banding (i.e., the age of 14–16 days). Because chick size varies with age, we omitted those few broods where the nestlings were measured before or after 14–16 days of nestling age ($n = 30$); thus we had $n = 674$ broods for nestling size analyses.

For analysing the effects of weather on nestling mortality, we used broods in which at least one offspring was alive on the third day after the hatching of the first nestling. We excluded a small number of broods ($n = 7$) that failed within the first 3 days after hatching because the average interval between our nest monitoring visits was 3 days, so estimating the number of hatched chicks and their survival before the first nest check after hatching would be very uncertain. We also excluded those broods where chick mortality was likely unrelated to weather conditions ($n = 7$), i.e., occurred due to predation or human disturbance (i.e., recorded during the next observation after the occasion of capturing the parent bird on its nest). This yielded $n = 760$ broods for nestling mortality analyses, from which 385 had no chick mortality, 319 had partial mortality, and 56 had complete brood loss during the nestling period.

We calculated all weather variables (average temperature and number of hot days, detailed below) for the nestling period of each brood individually. For investigating nestling size, weather variables were calculated for the period from the day of hatching of the first chick to the day preceding the day of banding and measuring the nestlings. This sample included only broods where banding occurred at age 14–16 days (mean $\pm$ SE: 14.04 ± 0.03). For analysing nestling mortality, we calculated weather variables for the period from the day of hatching of the first offspring to either the day preceding the day of recording the last nestling's death or the day preceding the day of banding and measuring the nestlings (mean $\pm$ SE: 13.72 ± 0.07 , range: 3 – 18 days). We calculated the average temperature as the mean of hourly temperatures over the nestling period. There are several methods for quantifying extreme hot weather (Bailey and van de Pol, 2016), using various thresholds from 21 to 43°C depending on the geographic location. We calculated the number of hot

¹ www.asab.org

days during each nestling period using a threshold specific to our region. To calculate a threshold for hot days, first, we defined a reference period from the earliest hatching date to the latest chick banding date recorded in our 6-year data set (pooling all sites and years), which ranged from 9th April to 15th July. Then, to estimate the typical temperatures in our geographic area during this reference period, we used a 26-years dataset from an external reference weather station located in Szentkirályszabadja, a small village near Veszprém (47°57'06" É, 17°58'10" K, ca. 9.5 – 22 km from our study sites). This weather station is maintained by the Hungarian Meteorological Service and its temperature data are available from the National Oceanic and Atmospheric Administration database², with records of air temperature every 3 h (0, 3, 6, 12, 15, 18, 21 UTC) since 1993. Using this temperature dataset, we calculated the 90% percentile of all daily maximum temperatures for the reference period from this 26-year long dataset, which was 28.7°C. Finally, we calculated the number of hot days as the number of days when the daily maximum temperature was higher than the 28.7°C threshold during the nestling period for each brood. Fixed temperature thresholds for calculating hot days are widely used in ecological and evolutionary research (Bailey and van de Pol, 2016) based on the assumption of a fixed threshold for heat tolerance in adults and offspring (Anderson, 2006; Gardner et al., 2017; Marrot et al., 2017; McCowan and Griffith, 2021). Since there is no proper information about critical thermal tolerance values for great tit nestlings, we used the 28.7°C air temperature threshold to define hot days based on the logic that temperatures above this value have been rarely experienced (i.e., less than 10 % of time) by the studied populations over the latest decades (and given the long-term trend for climate warming, such high temperatures were likely even rarer during their earlier past).

Statistical Analyses

Analysing the effects of extreme weather is challenging. Extreme events are rare by definition, so their distribution is strongly skewed (for distribution of our variable, see **Supplementary Material, Section 1**). Furthermore, weather variables are often correlated with each other and with other variables influencing reproductive success (e.g., with calendar date), so multicollinearity can be a problem in models containing multiple predictors. Statistical methods that can handle multicollinearity, such as covariance-based structural equation modelling, are less well suited for handling non-normal residual distributions and the non-independence structure of ecological data (e.g., multiple broods per pair). Therefore, here we used general and generalized linear mixed-effects (LME and GLMM) models and Akaike's information-criterion (AIC) based model selection (Burnham and Anderson, 2002) to focus on the interaction between habitat type and the number of hot days in explaining reproductive variables. Mixed-effects models can be applied appropriately to non-normal and non-independent data, while model selection based on measures of the models' total explanatory power (model fit indices, like AIC) is not sensitive to multicollinearity (Graham, 2003).

²www.ncdc.noaa.gov

All analyses were run in R (version 4.0.0) (R Core Team, 2020). First, to examine the temperature characteristics of our study sites, we compared the number of hot days and the average temperature in the nestling periods (calculated separately for each brood) between urban and forest habitats. We constructed a generalized linear model with the generalized Poisson distribution to handle overdispersion for the number of hot days using "glmmTMB" package (Brooks et al., 2017), and a linear model for the average temperatures. In both models, study site (4 sites) and year (6 years) were the predictors. To statistically compare the mean of each meteorological variable between the two habitat types, we calculated marginal means from the models for each study site, then calculated a linear contrast for habitat comparison as the difference of the average of the two urban sites from the average of the two forest sites, using the "emmeans" R package (Lenth, 2018). We used this approach rather than including habitat type as a fixed effect and site as a random effect in the models because variance estimations of random effects with few levels are unreliable (Piepho et al., 2003; Bolker et al., 2008), whereas including both habitat type and site as fixed effects would have resulted in strong collinearity between these two factors (Dormann et al., 2013). Instead, we treated the four sites as if they were two control groups and two treatment groups in an experiment, and we used a pre-planned comparison to test the prediction that the two treatment (i.e., urban) groups would differ from the two control (i.e., forest) groups. Such pre-planned comparisons are a powerful approach for testing *a priori* hypotheses (Ruxton and Beauchamp, 2008).

To analyse average nestling mass and average tarsus length as response variables, we used the "lme" function of the "nlme" R package (Pinheiro et al., 2013) which assumes Gaussian error. For nestling mortality (proportion of hatched chicks that died by the age of 14–16 days) as the response variable, we used GLMM models using the "glmer" function of the "lme4" R package (Bates et al., 2015) with binomial error distribution and logit link function. All of our models contained pair identity as a random factor to control for the non-independence of broods produced by the same pair. Broods got the same pair ID if both the female and the male parents were the same, and a new pair ID was assigned to the brood when it had at least one different parent. Pairs where one or both parents were unidentifiable ($n = 43$ and 139 , respectively) got separate pair IDs (see **Supplementary Material, Section 1, Supplementary Table 1** for random effect of pair ID). There were 111 pairs of parents that had more than one brood in our dataset, ranging 2–6 broods/pair. Additionally, to handle overdispersion in models of nestling mortality, we used an observation-level random effect (Maindonald and Braun, 2010) and ran the models with the BOBYQA optimizer (Bates et al., 2015).

First, we built a simple model for each of the three response variables (nestling mass, tarsus length, and mortality) including only the number of hot days (used as a numeric covariate) and study site (used as a factor with four levels), and their two-way interaction as predictors. We carefully inspected all relevant diagnostics to ensure that model fit was adequate for all models (see **Supplementary Material, Sections 2, 3**). Then, to statistically compare the effect of hot days on nestling size and

mortality between the two habitat types, we calculated the slopes of the relationship between reproductive success measurements and the number of hot days for each study population, and we compared the average of the two urban slopes with the average of the two forest slopes using a linear contrast.

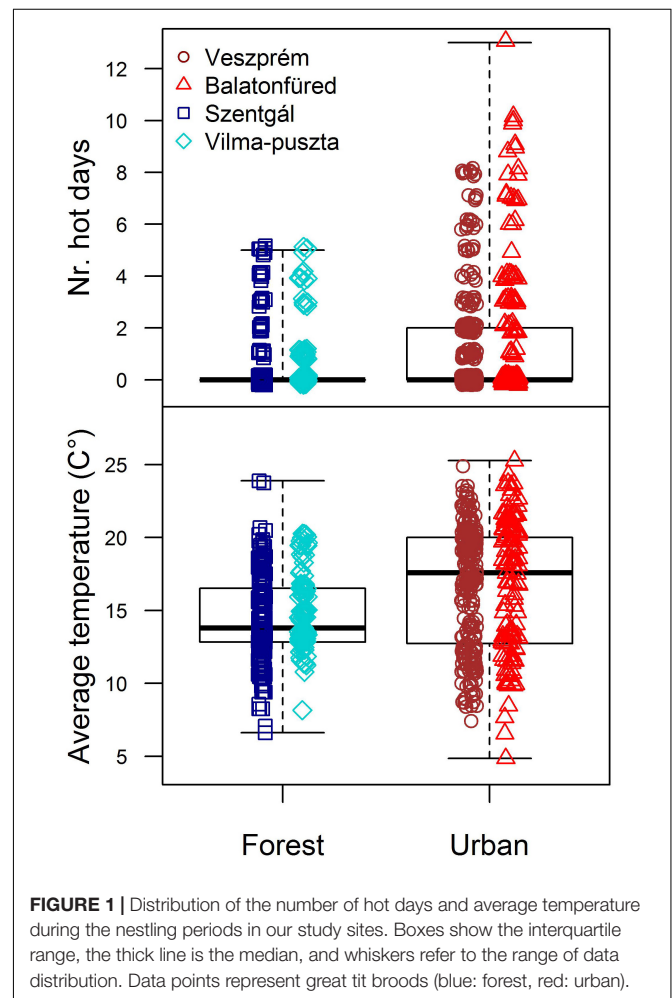
To infer the robustness of the results from the simple models, we also built multi-predictor models which included further potentially important predictor variables. In all models of our three response variables, we included year as a categorical variable, the average temperature during the nestling period and hatching date as numeric covariates, the latter defined as the number of days elapsed from the 1st January annually to the hatching of the first nestling in each brood. We also added the quadratic term of hatching date, because a preliminary inspection of diagnostic graphs suggested the possibility of non-linear seasonal changes in the response variables. For models of nestling characteristics (i.e., average body mass and average tarsus length of nestlings), we additionally included brood age (i.e., the number of days elapsed from the hatching of the first chick to the day of nestlings' measuring, ranging from 14 to 16 days), and brood size (i.e., the number of offspring alive at measuring). We standardized all numeric predictors using z-transformation for our multi-predictor analyses to avoid model convergence problems due to different scales of variables. Based on variance inflation factor (VIF), we detected high levels of multicollinearity in these multi-predictor models (see **Supplementary Material, Section 4, Supplementary Table 2**). Because multicollinearity with VIF values > ca. 2 can lead to unreliable standard errors, non-significant parameter estimates from such models must be treated with caution (Graham, 2003). Therefore, such models cannot be used for quantifying the effects of each predictor: for example, the number of hot days systematically increases with date, so the effects of hot days and other seasonal changes cannot be separated or compared by using our observational data. However, the set of predictors that yields the best explanatory power can be correctly identified by model selection based on model fit statistics (like AIC), even if multicollinearity is present (Burnham and Anderson, 2002). Therefore, we compared multi-predictor models to test if inclusion of any predictor increases model fit considerably, and then to see if our interaction of interest (urbanisation $\times$ number of hot days) is qualitatively altered by taking into account those extra predictors. We created a candidate model set for each response variable using all combinations of predictors but always including the number of hot days, study site, and their interaction (because this interaction was the focus of our study, and our goal with the model-selection procedure was to see if this interaction yields similar habitat contrasts in the multi-predictor models as alone in the simple models). The model set contained 33 models for nestling size (**Supplementary Material, Section 5, Supplementary Tables 3, 4**) and 9 models for nestling mortality (**Supplementary Material, Section 5, Supplementary Table 5**). We compared the models in each model set based on their AICc (AIC corrected for sample size) to identify the most supported model(s) for each response variable (Burnham and Anderson, 2002) using the "model.sel" function of the "MuMIn" R package (Barton, 2009). From the model(s) with superior support based

on $\Delta AICc$ and Akaike weight, we calculated the slope for each study site and the linear contrast that compares the effect of hot days between urban and forest sites the same way as from the simple models.

RESULTS

Habitat Differences in Heat Events

Great tit offspring experienced markedly different temperature characteristics in urban and forest sites during the nestling periods (**Figure 1**). The incidence of hot days occurring during the nestling period was 2.7 times greater in the cities than in the forests (urban-forest difference on log scale, controlled for year effects: mean $\pm$ SE: 0.99 ± 0.13 , $t = 7.39$, $df = 750$, $p < 0.001$; **Figure 1**). At least one hot day occurred in 179 out of 390 (45.9%) urban broods and in 77 out of 370 (20.8%) forest broods. The number of hot days ranged 0–13 in urban broods, taking up a maximum of 86.7% of the total length of individual nestling periods. In contrast, forest broods experienced only 0–5 hot days, which made up a maximum of 35.7% of the nestling period (see **Supplementary Material**,



Section 1 for further details). The highest maximum temperature was 40.1°C in urban nestling periods (in June 2013) and 33.6°C in forest nestling periods (in May 2014). Hot days were often on consecutive days during the nestling periods: there were 197 broods where at least two hot days occurred, and in 107 of these broods all hot days were consecutive (out of these 107 cases of “heatwaves,” 71 were at least 3 days long). The average temperatures within the nestling periods were significantly higher in the urban habitat (mean $\pm$ SD: 16.62 $\pm$ 4.29°C) than in the forest habitat (14.55 $\pm$ 2.90°C; urban-forest difference controlled for year effects: 1.52 $\pm$ 0.27°C, $t = 5.74$, $df = 751$, $p < 0.001$; **Figure 1**).

Effects of Hot Days on Reproductive Success

Nestling Body Mass

In the simple model where predictors were the number of hot days, study site and their two-way interaction, the effect of hot days on nestling mass differed between sites (as indicated by a significant interaction between sites and number of hot days, **Table 1**) and between urban and forest habitats (according to the linear contrast *post hoc* test comparing the effects between the two urban and the two forest populations, **Table 2**): increase in the number of hot days negatively affected nestling mass in forest broods while no such trend was present for the urban broods (**Figure 2A**). This result is consistent with the site-specific trends: the two forest populations had the most negative slopes for the relationship of nestling mass with the number of hot days, whereas the slope was flat or even positive in the two urban populations (**Supplementary Material, Section 6, Supplementary Table 6**). The habitat difference in heat effect remained significant when the analysis was restricted to the range of the number of hot days that occurred in both habitats, i.e., to broods with a maximum of 5 hot days (**Supplementary Material, Section, Supplementary Table 7**).

We also tested whether the effect of hot days persist when further potentially important variables (i.e., hatching date, year, brood size and brood age) are taken into account. We built a set of models containing various combinations of these variables in addition to the number of hot days, study site and their interaction, and we compared these models using the information-theoretic approach based on AICc. Model selection for these multi-predictor models resulted in two supported models for nestling mass ($\Delta AICc < 2$; the model with the third best fit had $\Delta AICc = 4$; **Supplementary Table 3**). The first supported model included year, brood age, and brood size besides the number of hot days, study sites, and their interaction; the second supported model contained the same predictors except brood size. Linear contrasts calculated from each of these supported multi-predictor models showed a significant overall habitat difference (**Table 2**) with a more negative effect of hot days in forest compared to urban populations (**Supplementary Table 6**), corroborating the results of the simple model.

Nestling Tarsus Length

In the simple model, the interaction between site and number of hot days was significant (**Table 1**), due to the difference

between the two urban sites, with a positive slope in one site and negative in the other (**Supplementary Table 6**). The two forest sites both showed negative relationship between the number of hot days and nestling tarsus length (**Supplementary Table 6**). The linear contrast for urban-forest difference suggested no systematic habitat difference in the effect of hot days on the tarsus length of nestlings (**Table 2** and **Figure 2B**).

Model selection for the multi-predictor model set resulted in a single supported model, which contained the number of hot days, study site, their interaction, the average temperature of the nestling period, brood age, and brood size (for the other models $\Delta AICc \geq 6.68$, **Supplementary Table 4**). The result of linear contrast comparing urban and forest slopes from this model was qualitatively identical to the result from the simple model, showing no consistent habitat difference in the effect of hot days on nestling tarsus length (**Table 2** and **Supplementary Table 6**).

Nestling Mortality

The simple model showed that the effect of the number of hot days on nestling mortality differed significantly between study sites (**Table 1**). The slope of the relationship between nestling mortality and the number of hot days was positive in all populations, being highest in one of the forest sites and lowest in one of the urban sites (**Supplementary Table 6**). The slopes also differed between urban and forest populations with a stronger effect in the latter (**Table 2** and **Figure 2C**), i.e., nestling mortality increased significantly more with the number of hot days in the forests than in the cities. The habitat difference in heat effect remained significant when the analysis was restricted to broods with a maximum of 5 hot days (**Supplementary Table 7**).

Multi-predictor model selection resulted in a single supported model, which was the full model of our model set excepting the quadratic hatching date (for the other models $\Delta AICc \geq 42.87$, **Supplementary Table 5**), containing the number of hot days, study site and their interaction, the average temperature of the nestling period, year and hatching date. The linear contrast calculated from this model showed a significantly steeper slope in forests compared to urban sites (**Table 2**), corroborating again the result of the simple model.

DISCUSSION

Our study found clear differences between urban and non-urban populations of an urban exploiter passerine species in the effects of hot days on important fitness components. Although the influence of thermal environment on fitness has been shown to vary with habitat urbanisation in ectotherms (Martin et al., 2021), our study is the first to demonstrate such habitat-dependent responses to heat stress in an endothermic animal. While UHI caused a 1.19°C higher temperature on average and there were more hot days in the cities compared to forests (**Supplementary Figure 1**), hot days affected nestling mass and survival more negatively in the forest habitat type. These results are robust, as they are based on >600 broods from 6 years and four sites, and were qualitatively unchanged by taking into account several potentially confounding factors that may differ between urban

and forest habitats, such as the start of breeding and brood size. These findings highlight that both lethal and sublethal effects of heat events (i.e., the proportion of surviving offspring and their quality in terms of body mass) can differ between urban and non-urban habitats. Although information about sublethal fitness costs of hot weather is scarce (Conradie et al., 2019), developing under hot weather conditions may have long-term consequences for fitness. Our results thus support the “adaptation to UHI” hypothesis and suggest that non-urban populations of birds (and possibly other vertebrates) are more vulnerable to extreme heat than urban populations.

Heat may affect the reproductive output of birds in at least two ways: directly through offspring physiology and indirectly through food availability. Firstly, the negative effects of hot days on offspring mass and survival may emerge due to the direct physiological consequences of heat stress. Nestlings usually cannot maintain stable body temperature in the first few days of their lives, and their metabolic processes also differ from those of adults (Price and Dzialowski, 2018) leaving them potentially more vulnerable to extreme heat than adults. The heat responses of dependent offspring are poorly known, although in birds the nestling phase seems to be the most relevant period during ontogeny regarding heat effects compared to the incubation and post-fledging periods (Marques-Santos and Dingemanse, 2020). In hot environments, individuals can less effectively dissipate the excess heat as a consequence of increased metabolic rate, leading to hyperthermia. Increasing metabolism causes decreased utilization of food and faster mobilization of energy reserves, thus it may cause lower body mass. Additionally, during heat stress, evaporative water loss is elevated as the organism tries to cool itself by evaporation to maintain body temperature in the physiologically normal range, causing dehydration (Weathers,

1972; Arad et al., 1989). Thus, increased metabolic rate and water loss both can lead to decreased body mass, as it has been shown in several bird species (Quinteiro-Filho et al., 2010; Kruuk et al., 2015; Corregidor-Castro and Jones, 2021; Oswald et al., 2021). Although parents can help their nestlings thermoregulate, great tits cannot shade their nest-boxes and they are not known to be able to cool their nestlings in other ways such as providing water.

These direct physiological effects of heat might be better tolerated by urban animals due to changes in their physiological and morphological characteristics. For example, we found in the same study populations that urban great tit nestlings have fewer feathers and increased bare body surfaces than forest nestlings (Sándor et al., 2021), which may facilitate heat dissipation. Reduced body size may also help to cope with the heat in cities because animals with smaller size have a higher surface-biomass ratio which facilitates heat loss. Bergmann’s rule (Bergmann, 1847) predicts that reduced size is beneficial against dehydration and overheating in warmer environments (Weathers, 1972; but see Whitfield et al., 2015) such as cities. This idea is supported by urban-rural comparisons of invertebrate communities (Merckx et al., 2018), as well as by a study of water flea populations which found that urban individuals were generally smaller than rural ones, and smaller individuals were more heat tolerant (Brans et al., 2017). In birds, a study of white-browed scrubwrens (*Sericornis frontalis*) also found that smaller individuals survived better when more extreme hot and dry events occurred (Gardner et al., 2017). Urban birds are usually smaller than their non-urban conspecifics (Liker et al., 2008; Chamberlain et al., 2009; Caizergues et al., 2021) and this is also the case with the great tits in our study populations (Seress et al., 2018). Although experimental evidence shows that low availability of nestling food is a major cause of the reduced size of urban great tit nestlings

TABLE 1 | Relationship between the number of hot days during nestling development and nestlings’ body mass, tarsus length, and mortality, according to the simple models.

Model terms	Nestling mass			Nestling tarsus length			Nestling mortality		
	χ^2	df	p	χ^2	df	p	χ^2	df	p
Nr. hot days	3.119	1	0.077	6.134	1	0.013	5.799	1	0.016
Study site	243.518	3	<0.001	103.380	3	<0.001	91.078	3	<0.001
Nr. hot days × study site	29.289	3	<0.001	9.555	3	0.023	11.844	3	0.008

Type 3 ANOVA (analysis of deviance) table is shown for each model. Significant effects ($p < 0.05$) are highlighted in bold. For average nestling mass and tarsus length, the number of pairs was 535 and the number of broods was 674. For nestling mortality, the number of pairs was 600 and the number of broods was 760.

TABLE 2 | Differences between urban and forest habitats in the effect of hot days on nestling’s body mass, tarsus length, and mortality.

Response	Simple model				Multi-predictor model			
	contrast ± SE	df	t	p	contrast ± SE	df	t	p
Nestling mass	0.338 ± 0.085	135	3.978	<0.001	0.656 ± 0.166 / 0.720 ± 0.165	127 / 129	3.941 / 4.355	<0.001 / <0.001
Nestling tarsus length	0.046 ± 0.039	135	1.187	0.237	0.044 ± 0.075	127	0.590	0.556
Nestling mortality*	−0.408 ± 0.159	156	−2.564	0.010	−0.643 ± 0.281	149	−2.288	0.022

The table shows linear contrasts comparing the average slope of two urban versus two forest sites, calculated from the estimates of the simple models (left) and the most supported multi-predictor models (right) for each response variable. For nestling mass, results of two supported multi-predictor models are separated by “/”. Positive contrasts mean more positive slopes (i.e., less negative effects of hot days) in the urban habitat. Significant habitat differences ($p < 0.05$) are highlighted in bold.

* Estimates for nestling mortality are on the logit scale.

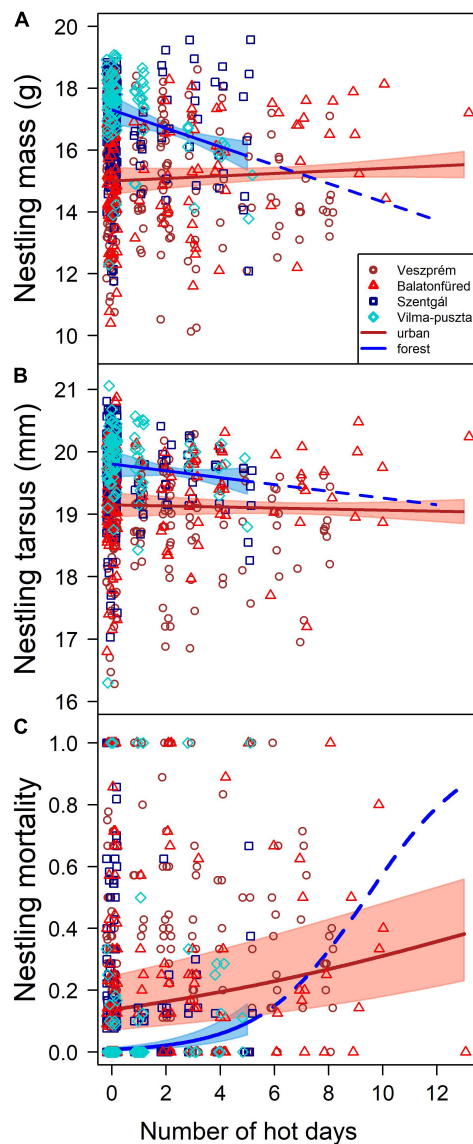


FIGURE 2 | Relationships between the number of hot days during the nestling period and **(A)** body mass, **(B)** tarsus length, and **(C)** mortality of great tit nestlings. Symbols represent brood means for nestling mass and tarsus length. Colored stripes show the 95% confidence band of the slope estimated from the simple models (**Supplementary Table 6**). For forest broods, the fitted slopes are extrapolated for the range of 5–13 hot days (dashed lines) using the slopes estimated by the models from the observed data. Note that all urban-forest comparisons remained qualitatively the same when the analyses were restricted to a subset of data where the number of hot days ranged from 0 to 5 (see **Supplementary Material, Section 7, Supplementary Table 7**).

(Seress et al., 2020), selection for better heat tolerance might also contribute to it. Finally, life-history changes may also help animals to cope with the direct effects of urban heat. For example, urban birds usually have smaller clutch size than forest birds (Chamberlain et al., 2009), which is also typical in our study populations (Seress et al., 2018, 2021). Smaller clutch size may

also be advantageous for heat dissipation when environmental temperature is high because fewer offspring produce less heat and have more space to maintain distance in the nest, which helps better heat conduction.

Secondly, indirect effects may also contribute to lower body mass and higher mortality in response to heat events. For example, hot weather may reduce food availability for insectivorous nestlings by affecting their parents and/or their prey. Caterpillars are the main source of nestling diet in great tits and many other birds, and increasing temperature strongly decreases the time to pupation in several lepidopteran species (Kingsolver et al., 1997; Lee and Roh, 2010; Lemoine et al., 2015), so optimal caterpillar food may be available for a shorter time in hot periods. Moreover, the growth of caterpillars declines rapidly above a critical temperature (Kingsolver et al., 1997) and their mortality increases when temperature is constantly high (York and Oberhauser, 2002; Lee and Roh, 2010). These heat effects may not only result in less food for nestlings but may also contribute to dehydration because caterpillars are the most water-rich items in the diet of nestlings (Zandt, 1997). These effects might be stronger in forests where the amount of available caterpillar prey is much higher in general (Seress et al., 2018) and the vast majority of nestling diet consists of caterpillars. In such environments, it might be more difficult for parent birds to compensate for a reduction in caterpillar biomass compared to cities, where nestlings are usually fed a greater proportion of other food types (Pollock et al., 2017; Jarrett et al., 2020). Water loss due to the combined effects of heat and reduced caterpillar availability might also be better compensated for in urban habitats if water is more accessible there, for example from public or garden ponds, bird water feeders, or puddles left behind after lawn irrigation. In our study, the nest-box colony in Balatonfüred is situated at ca. 700 m from Lake Balaton, and there is a small pond near some of our urban nest-boxes in Veszprém study site, while we are not aware of any permanent water sources in the forest study sites. However, we have no information on whether and how frequently the supplementary water sources are used by urban birds. Additionally, the foraging activity of parents may also decrease when temperature is high (Wiley and Ridley, 2016; Nilsson and Nord, 2018; Oswald et al., 2021), resulting in less food delivered to the offspring, and this effect might also be diminished in urban populations if parent birds have adapted to tolerate heat better (see above), or when they have more access to water than in non-urban forests. Further studies are required to test whether heat affects parental care differentially in urban and non-urban habitats.

Adaptation to constantly warmer urban environments may be achieved by microevolution, epigenetic changes, and/or phenotypic plasticity (Zhu et al., 2021). In some ectotherm species, artificial thermal selection, reciprocal transplant and common garden experiments have revealed that one or more of these mechanisms can play a role in the increased upper thermal tolerance of urban populations (Calosi et al., 2008; Brans et al., 2017; Campbell-Staton et al., 2020; Martin et al., 2021). To our knowledge, no such experiments have yet been done to identify the mechanisms of higher thermal tolerance in urbanised birds or other endothermic animals, which is an important knowledge

gap in our understanding of urban adaptations. Such experiments are also needed for quantifying the relative importance of heat *per se* and other environmental effects that may change over the season and may differ between urban and non-urban habitats, including parental investment and the availability of food and water, which was not possible with our correlative data here. Therefore, there is a great need for further studies that explore the differences in thermal tolerance between different habitats to understand how wild animals are affected by, and may adapt to, the interacting challenges of climate change and urbanisation in the Anthropocene.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be available from the authors upon request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Government Office of Veszprém County, Nature Conservation Division (former Middle Transdanubian Inspectorate for Environmental Protection, Natural Protection and Water Management; permission number: 24861/2014 and VE-09Z/03454-8/2018), and the National Scientific Ethical Committee on Animal Experimentation (permission number: VE/21/00480-9/2019).

AUTHOR CONTRIBUTIONS

IP, VB, and AL designed the study, did the statistical analyses, and wrote the manuscript. IP and VB made the data visualization. All authors collected data in the field and took part in finalizing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.825410/full#supplementary-material>

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Metabolic Profiling and Integration of Metabolomic and Transcriptomic Data From Pectoralis Muscle Reveal Winter-Adaptive Metabolic Responses of Black-Capped Chickadee and American Goldfinch

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Seasonal changes, such as alterations in food availability or type and cold conditions, present challenges to free-living birds living in highly seasonal climates. Small birds respond to such challenges through seasonal metabolic flexibility, which better matches seasonal metabolic phenotypes to environmental conditions and can improve fitness. To better understand the mechanistic basis of this metabolic flexibility, we conducted a large-scale metabolic profiling of pectoralis muscle in black-capped chickadees (*Poecile atricapillus*) and American goldfinches (*Spinus tristis*), which are small, year-round bird species of temperate-zones. We analyzed muscle samples using non-biased, global metabolomics profiling technology based on UHLC/MS/MS² platforms. A total of 582 metabolites was characterized for summer and winter season samples. Chickadees showed greater seasonal separation of global metabolite profiles than goldfinches, which is consistent with previous transcriptomic studies of pectoralis muscle in these two species. Reduced levels of amino acids during winter occurred in both species and might reflect decreasing dietary protein intake, amino acid shuttling to other pathways for thermogenesis and/or elevated rates of protein turnover in the pectoralis muscle. Concomitant decreased abundances in tricarboxylic acid cycle (TCA) metabolites suggest faster cycling of the oxidative phosphorylation pathway in winter to meet the metabolic demands of thermogenesis. Accordingly, chickadees displayed shifts toward lipid oxidation in winter, whereas goldfinches showed winter declines in ketone bodies, which suggests increased energy demand or subtle changes in substrate availability. Beyond the winter-specific changes in metabolite abundances, integration of the metabolomic and the transcriptomic data revealed a landscape of gene–metabolite associations related to the winter-adaptive metabolic response. This landscape of gene–metabolite pairs was overrepresented by pathways associated with transport of small molecules, metabolism of amino acids and derivatives, activation and biosynthesis of

fatty acid derivatives, and biosynthesis and metabolism of nicotinate and nicotinamide derivatives. Collectively, our results suggest that increased levels of NADH and its derivatives in the pectoralis muscle are a potential novel mechanism for increasing winter metabolic output, fueled by lipids, for thermogenesis during winter.

Keywords: phenotypic flexibility, birds, seasonal-adaptative response, metabolomics, winter, NAD⁺ metabolism, nicotinate and nicotinamide metabolism pathway

INTRODUCTION

Phenotypically flexible responses to season are ubiquitous among birds inhabiting seasonally variable climates, with those that live in cold winter climates adopting a strategy of metabolic upregulation and increased energy consumption in winter (Marsh and Dawson, 1989; Swanson, 2010). Cold winter climates typically result in upregulation of both basal (BMR) and summit (peak cold induced metabolic rates, M_{sum}) metabolic rates, although seasonal responses are more variable for birds in milder winter climates (McKechnie, 2008; Wells and Schaeffer, 2012; McKechnie et al., 2015). This seasonal metabolic flexibility allows birds to better match their metabolic phenotypes to prevailing environmental conditions (e.g., Vézina et al., 2020; Le Pogam et al., 2021). Because elevated M_{sum} in birds is associated with enhanced cold tolerance (Swanson, 2001; Swanson and Liknes, 2006), it represents a functional link between metabolic rates and performance that could influence survival over winter. Indeed, such matching of metabolic and cold tolerance phenotypes to seasonal conditions can have fitness consequences (Nilsson and Nilsson, 2016; Petit et al., 2017; Latimer et al., 2018).

The physiological, biochemical, and molecular mechanisms underlying metabolic flexibility in birds are incompletely understood (Cheviron and Swanson, 2017; Stager et al., 2021). Different bird species can employ or emphasize different mechanisms for generating elevated metabolic rates in cold winter climates (Liknes and Swanson, 2011a,b; Petit and Vézina, 2014; Petit et al., 2014; Dubois et al., 2016). In addition, within year-round temperate-zone resident populations, birds may show different metabolic and mechanistic responses to temperature variation across seasons (Swanson et al., 2020). Common pathways contributing to metabolic upregulation include muscle and digestive system growth, fat catabolism and transport capacities, cellular metabolic intensity, and oxygen transport capacity. However, seasonal adjustments to specific regulatory steps in each of these pathways is far from uniform among birds in cold winter climates (Swanson, 2010).

Because M_{sum} in birds is primarily a function of shivering in skeletal muscle, especially pectoralis muscle (Hohtola, 1982; Swanson et al., 2013; Petit and Vézina, 2014), flexibility in organismal M_{sum} may result from changes in muscle mass or cellular metabolic intensity (Swanson and Vézina, 2015). Winter increments of M_{sum} in birds are consistently supported by greater pectoralis muscle mass (Swanson and Vézina, 2015, but see Swanson et al., 2014; Milbergue et al., 2019) but less consistently by elevated cellular metabolic intensity [see Swanson (2010) for review; but also Liknes and Swanson (2011b), Peña-Villalobos et al. (2014), Zheng et al. (2014), Wang et al. (2019)].

Seasonal changes in the transport of oxygen and substrates (especially lipids, McWilliams et al., 2004; Guglielmo, 2010) also regularly contribute to seasonal metabolic variation in birds (Swanson, 2010; Petit and Vézina, 2014; Zhang et al., 2015a). Increases in lipid transport and catabolism are also often positively correlated with higher winter M_{sum} in birds (Swanson, 2010; Zhang et al., 2015b). Moreover, acute cold and exercise training increased lipid transport and cellular metabolic intensity in house sparrows, *Passer domesticus*, especially in terms of intracellular lipid transport *via* cytosolic fatty acid binding protein, FABP_c (Zhang et al., 2015c). Such changes are particularly important to birds because lipids serve as the primary fuel for shivering and birds rely on exogenous lipids to fuel this activity (McWilliams et al., 2004; Vaillancourt et al., 2005).

Metabolic flexibility and its mechanistic underpinnings are likely mediated, at least in part, through transcriptional changes in underlying gene expression networks (Ayroles et al., 2009; Stager et al., 2015; Cheviron and Swanson, 2017). Cheviron and Swanson (2017) studied seasonal transcriptomic variation pectoralis muscle, the primary thermogenic organ (Hohtola, 1982; Marsh and Dawson, 1989) in American goldfinches (*Spinus tristis*) and black-capped chickadees (*Parus atricapillus*), two North American year-round resident species in cold winter climates. Interestingly, chickadees showed greater summer-to-winter variation in differentially expressed genes than did goldfinches, although both species showed similar overall patterns of differential gene expression between seasons (Cheviron and Swanson, 2017). Some genes and pathways associated with muscle growth and remodeling showed differential seasonal expression in both chickadees and goldfinches (Cheviron and Swanson, 2017). The substrate metabolism pathways exhibiting significant increases in transcript abundance in winter included “fatty acid metabolism,” “tricarboxylic acid cycle,” and “oxidative phosphorylation” in chickadees and “glycerolipid metabolism” in both species (Cheviron and Swanson, 2017). These changes suggests that seasonal modifications to fat metabolism are an important component of winter acclimatization in both species.

The quantities and types of metabolites are downstream products of gene expression, protein interactions and other regulatory processes, and thus provide a functional snapshot of the phenotype under specific conditions (Guijas et al., 2018). Because gene expression does not necessarily indicate or correlate with protein expression or metabolic pathway regulation, including during seasonal acclimatization or temperature acclimation in birds (e.g., King et al., 2015; Stager et al., 2015; Zhang et al., 2015b), integration of ‘omics data sets can provide a robust, comprehensive picture of organismal

responses to changing energy demands that can reveal pathway-level changes that are not apparent through individual analytical methods (Tung et al., 2019; Savva et al., 2022). Few studies have attempted integration of different 'omics data sets. In the present study, however, we generated a metabolomics data set for the same individual birds from which Cheviron and Swanson (2017) developed a transcriptomic data set. We then integrated the metabolomic and transcriptomic data sets to compare black-capped chickadees and American goldfinches in summer and winter. We hypothesize that chickadees would show greater seasonal differences in metabolites relative to goldfinches, as they did for transcript abundances (Cheviron and Swanson, 2017). We also hypothesize that metabolites related to skeletal muscle function and growth and those that function in fat metabolism pathways will vary seasonally in a manner consistent with the increased abundances of related transcripts in winter in both species. We anticipate that integration of these 'omics data sets might also elucidate additional important mechanistic pathways that could contribute to seasonal metabolic phenotypes in small passerine birds.

MATERIALS AND METHODS

All birds for the present study were collected using mist nets near Vermillion, Clay County, South Dakota (approximately 42°47'N 96°55'W) in summer (June to August) and winter (December to February) in 2010–2012 as described in Swanson et al. (2014) and Cheviron and Swanson (2017). All American goldfinches used in this study (Summer $n = 10$, Winter $n = 7$) were adults. All black-capped chickadees collected in winter ($n = 7$) were adults, but summer-collected chickadees consisted of six adults and four fully grown independent hatch-year birds that were likely 1.5–2 months old (Cheviron and Swanson, 2017). Hatch-year summer chickadees did not differ significantly in body size or pectoralis muscle mass from adults (Cheviron and Swanson, 2017). After capture, we transported the birds back to the laboratory and euthanized them within 2 h by cervical dislocation. Following euthanasia, we rapidly dissected out pectoral muscle (one side only) on ice and weighed the sample to the nearest 0.1 mg before flash-freezing in liquid nitrogen. We stored muscle samples at -80°C until metabolomic assays, except that we briefly partially thawed the muscle sample to remove a sub-sample for another study (Swanson et al., 2014), but immediately re-froze the sample in liquid nitrogen after removal of the sub-sample. Some of the muscle samples did not have sufficient tissue for metabolic profiling, hence a slightly smaller subset was used compared to the transcriptomic set used in the Cheviron and Swanson (2017) study. The resulting tissue sample sizes used for profiling were Summer $n = 9$, Winter $n = 7$ for chickadees and Summer $n = 7$, Winter $n = 7$ for goldfinches, where transcriptomic data were available (Cheviron and Swanson, 2017) for these samples. We captured birds under active state (permit #10-2, #11-7, and #12-2) and federal (permit #MB758442) scientific collecting permits and all procedures were approved by the University of South Dakota Institutional Animal Care and Use Committee (Protocol # 79-01-11-14).

For metabolite extractions, we first ground pectoral muscle to a fine powder under liquid nitrogen and about 20 mg of powdered muscle was used for the unbiased global metabolic profiling performed by Metabolon (Durham, NC, United States). Briefly, each 20-mg muscle tissue sample was extracted at a 1:5 ratio in methanol plus recovery standards using an automated MicroLab STAR system (Hamilton Company, Salt Lake City, UT, United States). To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, and one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI.

When a metabolite was below the threshold of detection, data were imputed as the minimum detected quantity for that metabolite in this project. Resulting data were log (base 2)-transformed to normalize and then initially analyzed using a two-way ANOVA, with season and species as factors. Reported significant differences from the two-way ANOVA are $p < 0.05$ and $q < 0.10$, where q is the False Discovery Rate (FDR; Storey, 2002). We then mapped the detected metabolites onto the biochemical pathways to which they belonged and categorized pathways into related groups (e.g., glucose mapped onto the glycolysis pathway and grouped into carbohydrate metabolism). We used principal component analysis (PCA) to transform the detected metabolites into a smaller number of orthogonal variables (i.e., component 1 and component 2) to visualize the overall variation in metabolite profiles among species and seasons and to provide a global overview of the dataset.

We used the web-based metabolomics data processing tool, MetaboAnalyst 5.0¹ to provide overviews of metabolite data (i.e., fold-change analysis and comparative cluster analysis). Briefly, univariate fold-change analyses were performed using the fold-change analysis module for each species with fold-change threshold set at 2.0. For an overview of hierarchical cluster analysis (HCA), a heat map visualization of metabolite abundances was generated from hierarchical clustering using Ward's linkage and Euclidean distance. We focused our interpretations of metabolite analyses on substrate (carbohydrates, amino acids, and lipids) metabolism pathways and on intermediates of key metabolic pathways related to energy production [e.g., tricarboxylic Acid Cycle (TCA), glycolysis, and NAD/NADH metabolism], which are relevant to previously documented seasonal variation in organismal metabolic capacities in the two study species (Cooper and Swanson, 1994; Liknes et al., 2002).

To evaluate whether the relationships between transcript and metabolites differ by species and season, we used a linear model approach (Siddiqui et al., 2018) to integrate the current metabolomics data with the previously published transcriptomics data for the same tissues of black-capped chickadees and

¹<https://www.metaboanalyst.ca/>

American goldfinches (Cheviron and Swanson, 2017). This linear model approach can identify transcript-metabolite associations that are specific to a particular phenotype (Patt et al., 2019; Banerjee et al., 2020). Significant transcript-metabolite pairs were clustered by the direction of association (i.e., Winter correlated and Winter anti-correlated). Winter correlated means that certain combinations of both transcripts and metabolites increase in winter relative to summer, whereas Winter anti-correlated means that certain combinations of transcripts increase and metabolites decrease (or vice versa) in winter relative to summer. To reveal the underlying seasonal pathways in each species (i.e., Winter-specific and Summer-specific) from the integration of the metabolomic and transcriptomic data, we conducted pathway overrepresentation analyses on significant transcript-metabolite pairs using the web-based 'omics integration tool, IMPALA: Integrated Molecular Pathway Level Analysis (Kamburov et al., 2011)².

RESULTS

Metabolome Composition of Black-Capped Chickadee and American Goldfinch

A total of 582 metabolites was characterized in samples from birds collected in summer or winter to determine patterns of seasonal shifts in metabolites of black-capped chickadees and American goldfinches (Figure 1A). Lipids (50%) were the most prevalent, followed by amino acids (21%), carbohydrates (8%), nucleotides (7%), cofactors (5%), peptides (4%), and xenobiotics (3%). Energy-related metabolites were the least (2%) prevalent. PCA indicated that these samples formed distinct groupings by species and season, where Component 1 separated the two species and Component 2 separated between Summer and Winter samples (Figure 1B). Chickadees showed the greatest separation between the Summer and Winter samples, whereas goldfinches showed much less separation between Summer and Winter samples. HCA indicated a primary split in the bottom third of the heatmap that separated chickadee Winter samples from Summer samples, with a secondary split separating goldfinch Winter samples from Summer samples (Figure 2A).

Seasonal Shifts in Metabolic Profiles of Pectoral Muscle

More distinct significant changes were detected in the metabolic profiles of pectoral muscles of chickadees between Summer and Winter compared to pectoral muscles of goldfinches between Summer and Winter (Figure 2A). Specifically, 198 metabolites exhibited more than a twofold significant change in abundance between Summer and Winter profiles in chickadees, whereas only 85 metabolites exhibited more than a twofold significant change in abundance between Summer and Winter profiles in goldfinches (Figures 2B,C and Supplementary Tables 1, 2). In winter, chickadees showed greater than twofold enrichment

in arginine and proline metabolism, pyrimidine metabolism, arginine biosynthesis, pantothenate and CoA biosynthesis, nicotinate and nicotinamide metabolism, and histidine metabolism, whereas goldfinches showed greater than twofold enrichment in taurine and hypotaurine metabolism, arginine biosynthesis, D-glutamine and D-glutamate metabolism, lysine, deregulation, sulfur metabolism, biosynthesis of unsaturated fatty acids (FAs), and alpha-linolenic metabolism (Figures 2D,E).

Seasonal Shifts in Amino Acids

The abundance of amino acids tended to decrease in Winter for both species. Abundances of several amino acids (e.g., Ala, alanine; Glu, glutamate; His, histidine; Lys, lysine; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Leu, leucine; Ile, isoleucine; Val, valine; Met, methionine; Arg, arginine) and their catabolites tended to decrease in Winter, though these shifts did not achieve significance for comparisons in either species (Figure 3A). While Arg abundance tended to decrease in Winter, creatine abundance was elevated in Winter for both species, with a significant increase in creatinine only for chickadees. The abundance of creatine phosphate decreased in Winter for both species (Figure 3B) but did not vary significantly with season in goldfinches.

Seasonal Shifts in Lipid Metabolites

Fatty acids are a critical source of energy for mitochondrial oxidation and cellular ATP generation, as well as being precursors for phospholipids and storage lipids. While the abundance of long-chain FAs did not significantly differ between Winter and Summer for goldfinches (Figure 4), the abundance of longer-chain FAs (e.g., C20-C24 FAs behenate, erucate, and nervonate) was increased in Winter for chickadees, whereas the abundances of several other FAs (e.g., myristate, margarate, and 1-heptadecenoate) decreased. Interestingly, the abundance of omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoate and docosapentaenoate were also lower in Winter for both species, whereas the abundance omega-6 PUFAs (e.g., linoleate, dihomo-linoleate) were elevated in Winter for chickadees. Also, the abundance of 1-stearoyl-2-arachidonoyl-GPC tended to increase in Winter for both species.

Seasonal Shifts in Carbohydrate Metabolites

Few significant changes in carbohydrate metabolism were detected in Winter for goldfinches. However, the abundance of 3-carbon glycolytic intermediates (2-phosphoglycerate, 3-phosphoglycerate, and phosphoenolpyruvate) and pyruvate decreased in Winter chickadees but not goldfinches (Figure 5).

Seasonal Shifts in Tricarboxylic Acid Cycle Metabolites

Carbohydrates and lipids are among several sources of carbon for the TCA cycle. Other carbon sources could include, *via* conversion of acetyl-CoA to citrate, glutamine entering as alpha-ketoglutarate, and branched-chain amino acids entering as citrate and succinyl-CoA. Decreased abundances of citrate,

²<http://impala.molgen.mpg.de/>

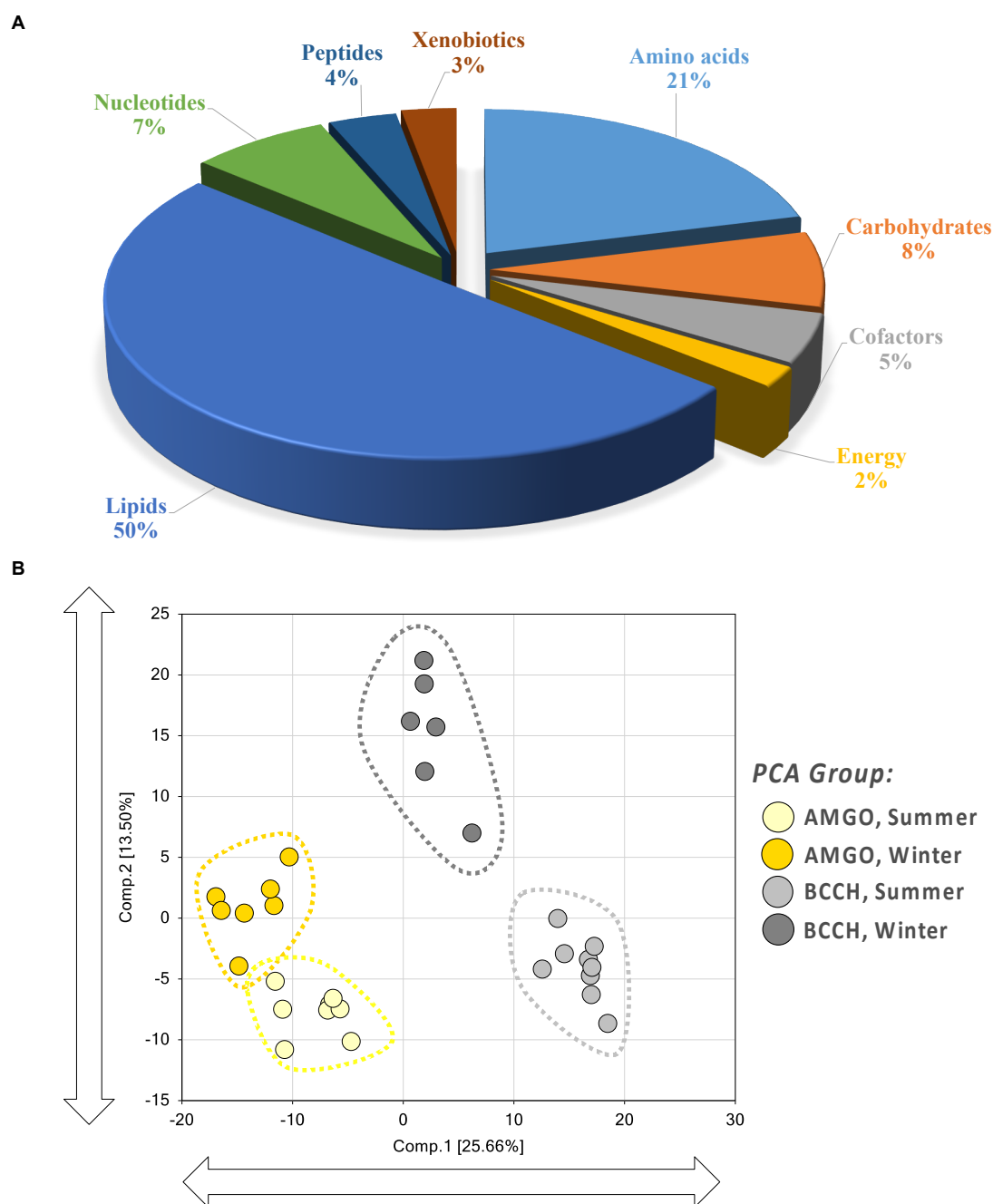


FIGURE 1 | Global metabolome of pectoralis muscle in Black-capped Chickadee (BCCH) and American Goldfinch (AMGO) during Summer and Winter.

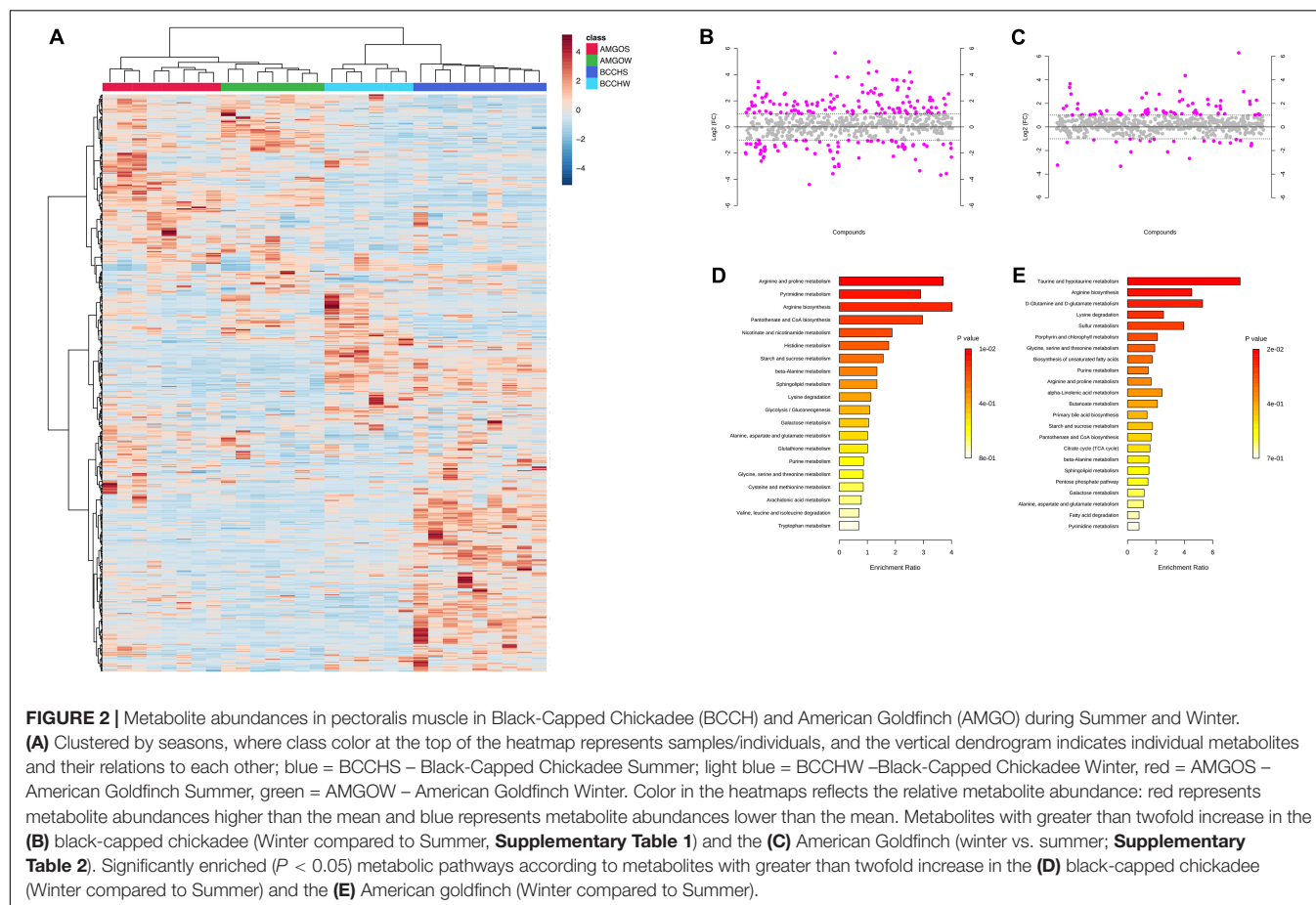
(A) Functional categorization of 582 identified metabolites detected. **(B)** Pectoralis muscle sample distribution as determined by Principal Component Analysis (PCA). Ovoids highlight species/season combinations.

isocitrate, and alpha-ketoglutarate were observed in Winter for goldfinches and the abundance of succinate decreased in Winter for chickadees (**Figure 6**).

Seasonal Shifts in Other Metabolites

The abundance of several metabolites showed trends toward increasing levels in Winter for one or both species. The

abundance of tocopherols increased in Summer for both species, whereas the abundances of biliverdin, taurocholate, and taurochenodeoxycholate were elevated in Winter only for goldfinches. The abundances of serotonin, and tryptophan betaine increased in Winter, but only for chickadees (**Figure 7**). The abundance of NADH was significantly increased in Winter for both species, whereas the abundances of nicotinamide



riboside was significantly elevated in Winter only for goldfinches (Figure 8).

Landscape of Gene-Metabolite Associations

Changes in season-specific transcript-metabolite pairs were much more pronounced in black-capped chickadee compared to American goldfinch (Figure 9). Linear model analysis identified 3,019 Winter-specific transcript-metabolite associations in chickadees (Benjamini-Hochberg FDR, Benjamini and Hochberg, 1995; adjusted interaction p -value < 0.10 , and a Spearman correlation difference $> |\pm 0.5|$, **Supplementary Table 3**) involving 1,651 genes and 269 metabolites. In contrast, the analysis identified only 69 Winter-specific transcript-metabolite associations in goldfinches (Benjamini-Hochberg FDR- adjusted interaction p -value < 0.10 , and a Spearman correlation difference $> |\pm 0.5|$; **Supplementary Table 4**) involving 53 genes and 38 metabolites. Clustering of these pairs by the positive (i.e., Winter correlated) or negative (i.e., Winter anti-correlated) direction of association within each season revealed two major clusters for both chickadees (Figure 9A) and goldfinches (Figure 9B). For chickadees, the Winter correlated cluster of 1,373 transcript-metabolite pairs (816 unique genes and 185 unique metabolites) showed

relatively high positive correlations in Winter samples and low or mostly negative correlations in Summer samples, whereas the Winter anti-correlated cluster of 1,646 transcript-metabolite pairs (908 unique genes and 180 unique metabolites) showed relatively high negative correlations in Winter samples and positive or low negative correlations in Summer samples (**Supplementary Table 3**). In contrast, the Winter correlated cluster in goldfinches consisted of 26 transcript-metabolite pairs (20 unique genes and 18 unique metabolites) with relatively high positive correlations in Winter samples and mostly negative correlations in Summer samples. The Winter anti-correlated cluster in goldfinches was composed of 43 transcript-metabolite pairs (33 unique genes and 28 unique metabolites) with relatively high negative correlations in Winter samples and relatively high positive correlations in Summer samples (**Supplementary Table 4**).

Integrated molecular pathway level analysis of the 1,373 transcript-metabolite pairs (mappable genes and metabolites) in the chickadee Winter correlated cluster identified 1,065 pathways that were overrepresented (**Supplementary Table 5**), with 781 pathways shared with the Winter anti-correlated cluster and 284 pathways unique to the Winter correlated cluster. In the Winter correlated cluster (i.e., 1,065 pathways), the top 25 overrepresented transcript-metabolite pathways fall within pathways associated with transport of small molecules,

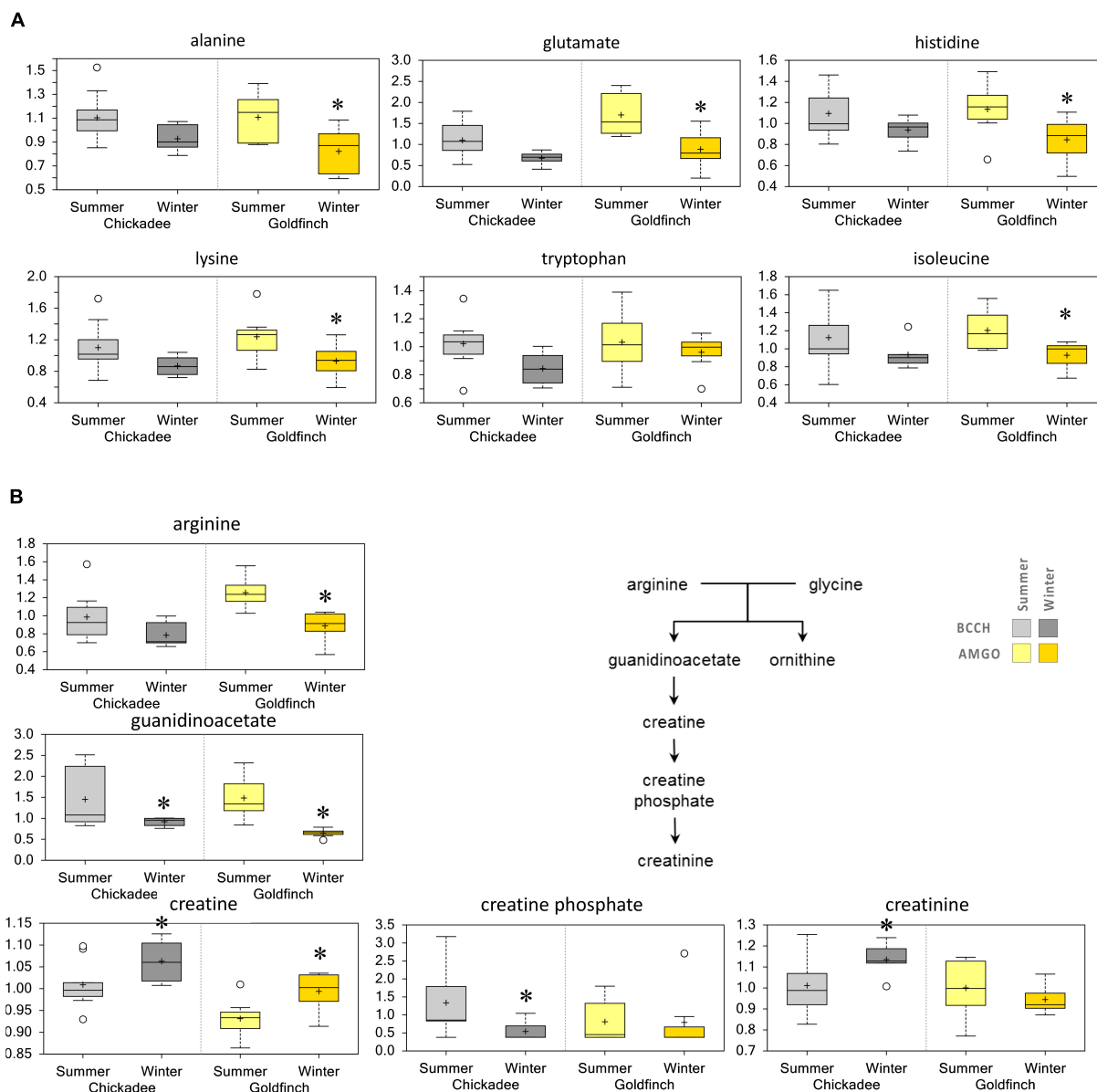
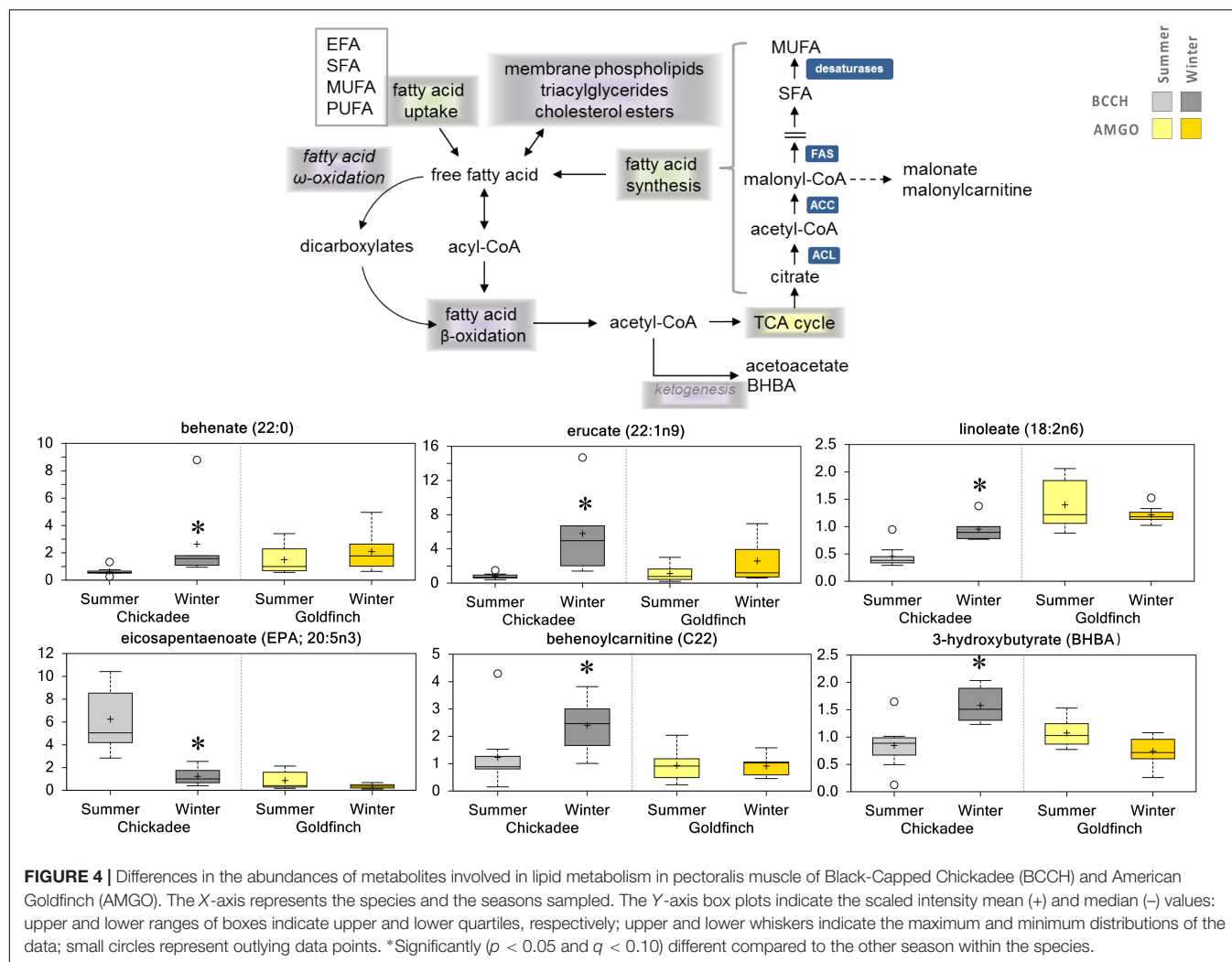


FIGURE 3 | Differences in the abundances of **(A)** amino acids and **(B)** amino acid derivatives in pectoralis muscle of Black-Capped Chickadee (BCCH) and American Goldfinch (AMGO). The X-axis represents the species and the seasons sampled. The Y-axis box plots indicate the scaled intensity mean (+) and median (–) values: upper and lower ranges of boxes indicate upper and lower quartiles, respectively; upper and lower whiskers indicate the maximum and minimum distributions of the data; small circles represent outlying data points. *Significantly ($p < 0.05$ and $q < 0.10$) different compared to the other season within the species.

metabolism of amino acids and derivatives, and activation and biosynthesis of fatty acid derivatives. The top 25 unique (i.e., 284 pathways) overrepresented transcript-metabolite pathways included beta oxidation of FAs and biosynthesis of amino acids and carnitine derivatives (**Supplementary Table 9**). Of the chickadee Winter anti-correlated cluster transcript-metabolite pairs ($N = 1,686$), 983 pathways were overrepresented (**Supplementary Table 6**) and 202 pathways were unique to the Winter anti-correlated cluster. Like the Winter correlated cluster, the top 25 overrepresented transcript-metabolite pathways of the Winter anti-correlated cluster (i.e., 983 pathways)

included pathways associated with transport of the small molecules, metabolism of amino acids and nucleotides, and biosynthesis of FAs. The top 25 unique (i.e., 202 pathways) overrepresented transcript-metabolite pathways in chickadees included communication and release of neurotransmitters and related signaling molecules (**Supplementary Table 9**). Interestingly, overrepresented transcript-metabolite pathways common to both Winter correlated and anti-correlated clusters are metabolism of polyamines, and biosynthesis of putrescine, spermine, and spermidine, as well as their precursor ornithine (**Supplementary Tables 5, 6**).

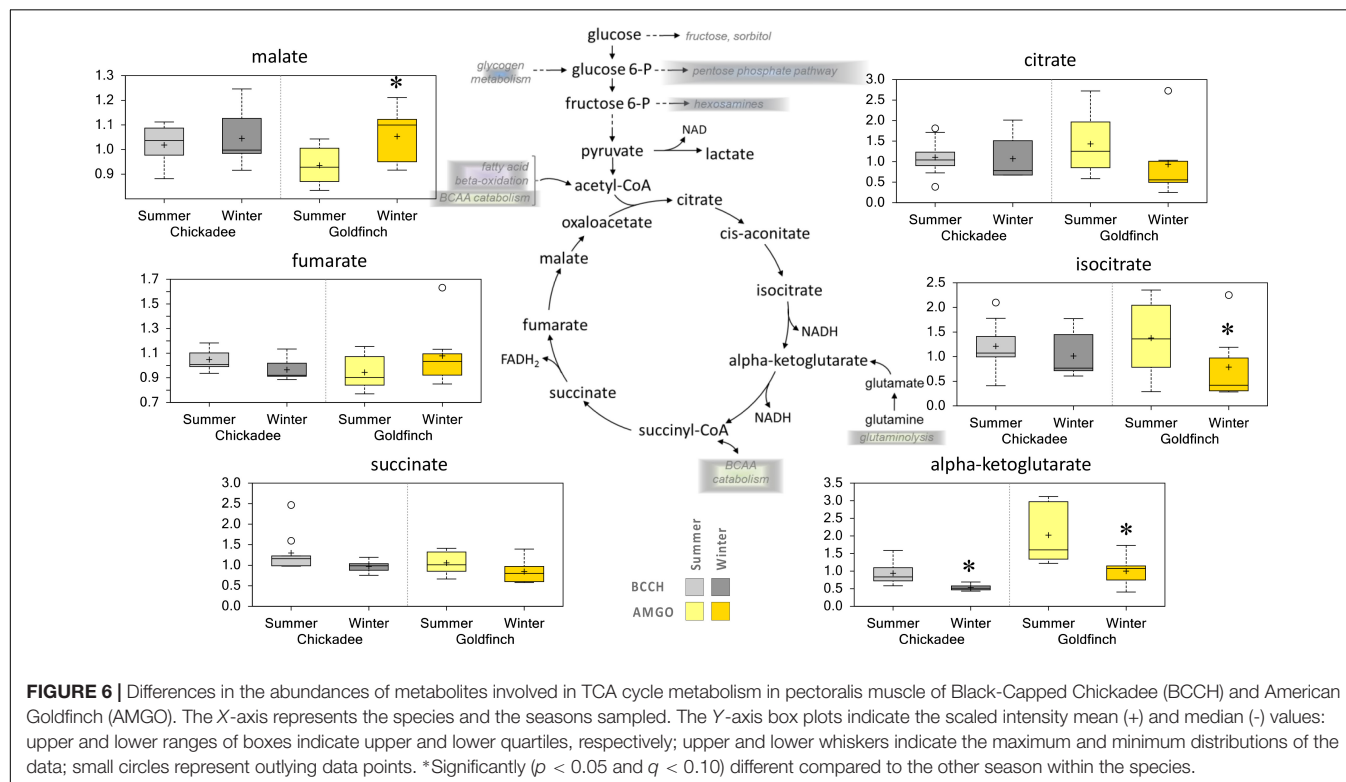
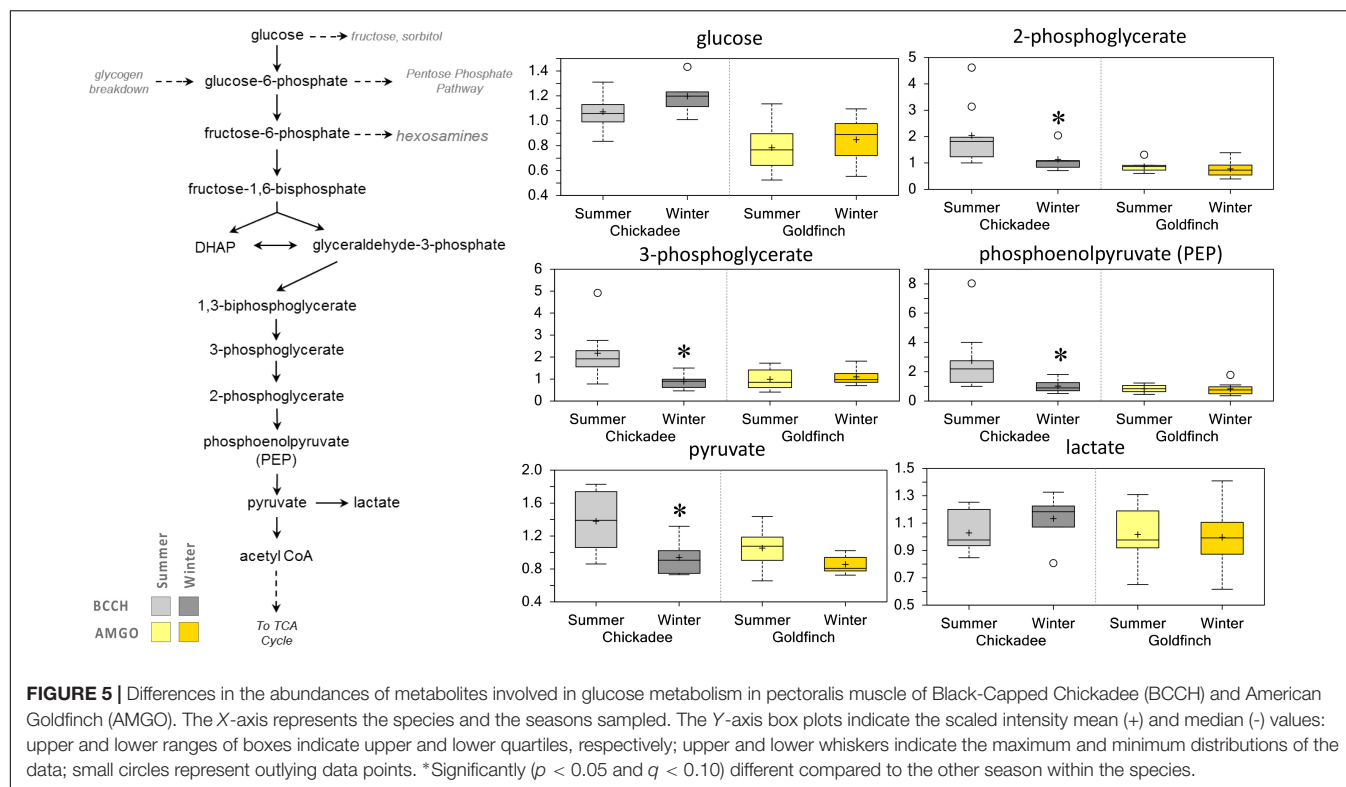


In contrast to chickadees, integrated molecular pathway level analysis of the 26 transcript-metabolite pairs (mappable genes and metabolites) in the goldfinch Winter correlated cluster identified 40 pathways that were overrepresented (Supplementary Table 7). Of these 40 pathways, 28 were shared with the Winter anti-correlated cluster and 12 pathways were unique to the Winter correlated cluster. In the Winter correlated cluster (i.e., 40 pathways), the top 25 transcript-metabolite pathways overrepresented included metabolism of sphingolipids and osteoblast signaling. Only 12 unique overrepresented transcript-metabolite pathways were uncovered and these mainly included metabolism and biosynthesis of nicotinamides (Supplementary Table 10). Of the goldfinch Winter anti-correlated cluster of transcript-metabolite pairs ($N = 43$), 96 pathways were overrepresented (Supplementary Table 8), with 68 pathways unique to the Winter anti-correlated cluster. In the Winter anti-correlated cluster (i.e., 96 pathways), the top 25 transcript-metabolite pathways overrepresented include transport of nucleotides and vitamins, metabolism of sphingolipids and lipids. Overall, the top 25 unique (i.e., 68 pathways) transcript-metabolite overrepresented pathways

include transport of nucleosides and vitamins and metabolism of nucleotides and FAs (Supplementary Table 10).

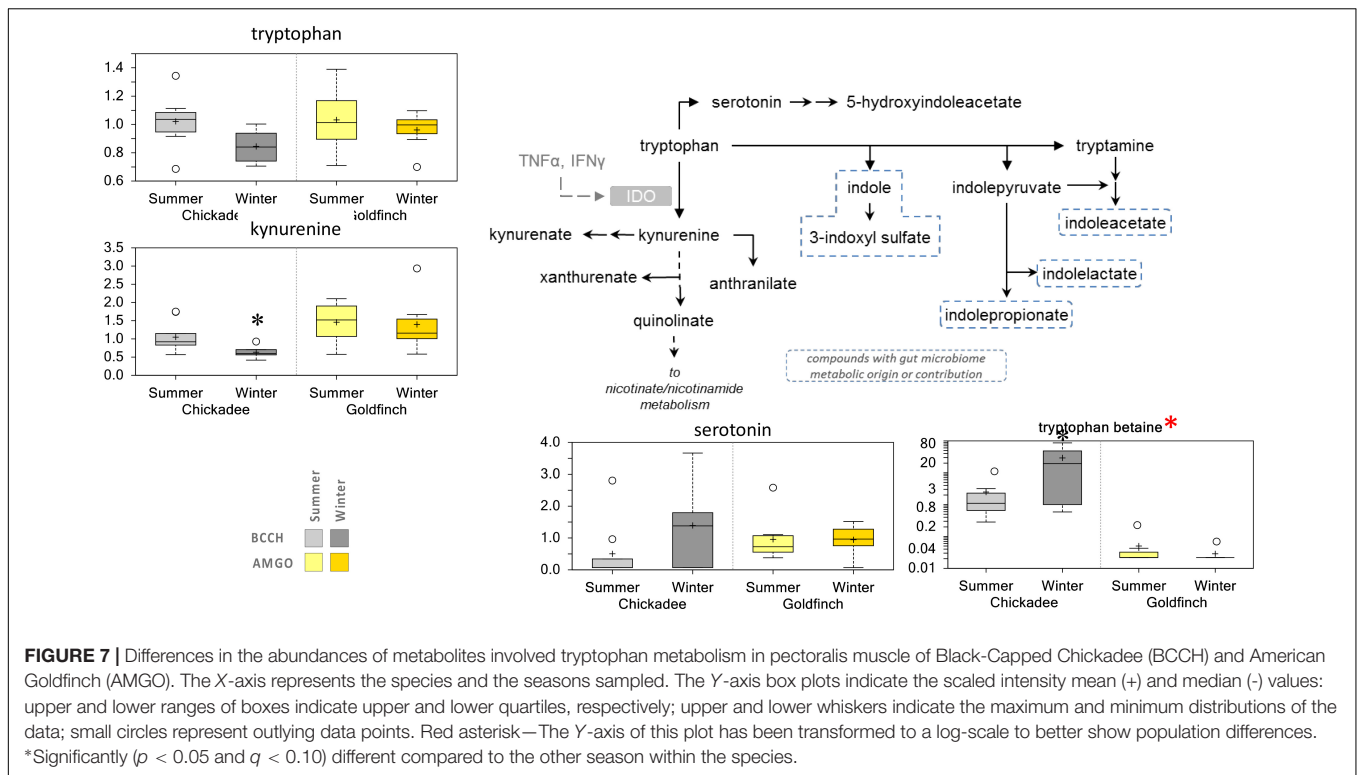
DISCUSSION

The winter-adaptive response of small birds is a complex phenomenon influenced by environmental and genetic factors and involves many molecular/biochemical pathways in the primary thermogenic organ, the pectoralis muscle. Because the pectoralis muscle is also the largest organ in volant birds and is important to both locomotion and shivering, the pectoralis muscle is also an essential location for the metabolism of carbohydrates and lipids (Pedersen and Febbraio, 2012; Morales et al., 2017). Our metabolic profiling and integration of the metabolomic and transcriptomic data for the same muscle tissue not only highlighted molecular functions and pathways linked to the winter-adaptive response but revealed previously unknown winter-specific patterns of transcript-metabolite coordination that could contribute to the metabolic flexibility of temperate-zone resident birds during cold winter seasons.



The abundances of some complex polyunsaturated lipids (e.g., 1-stearoyl-2-arachidonoyl-GPC) tended to increase in both chickadees and goldfinches in Winter compared with Summer.

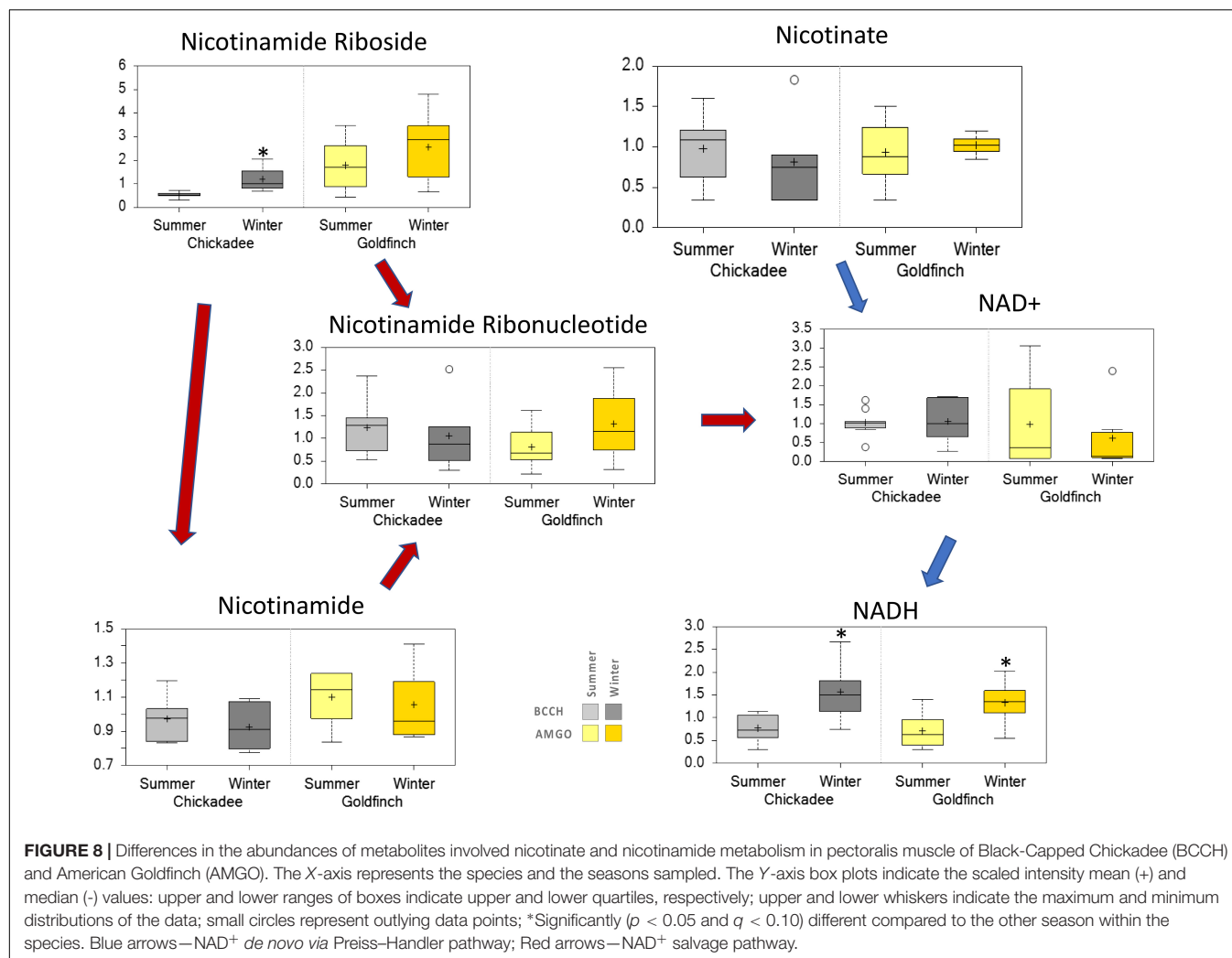
In addition, omega-3 PUFAs tended to show decreases in winter, whereas omega-6 PUFAs increased in winter for chickadees. These differences could reflect seasonal differences in dietary



intake. Goldfinches are thought to eat primarily a vegetarian diet year-round (McGraw and Middleton, 2020), and whereas chickadees eat predominantly insects in summer, their diet is supplemented with seeds and fruit in winter when insects are scarce (Foote et al., 2020). Seeds tend to be high in oleate and linoleate and other PUFAs (Collins and Horn, 2012; Maikhuri et al., 2021), whereas insects such as caterpillars can be high in omega-3 PUFAs (Lautenschläger et al., 2017; Guil-Guerrero et al., 2018; Mba et al., 2019). In addition, aquatic insects may represent an important dietary subsidy for woodland birds (Smith et al., 2007; Allen, 2019), including chickadees and goldfinches, if woodlands are located near water bodies, as many of our study sites were. Aquatic arthropods contain highly unsaturated omega-3 fatty acids from their algal-based diet and, therefore, differ from most terrestrial arthropods in fatty acid composition of body tissues (Twining et al., 2018, 2019, 2021). The low dietary intake of arthropods, especially aquatic insects, in winter by both species could potentially help explain reduced levels of dietary PUFAs and this could potentially impact metabolic performance. However, woodland birds like chickadees and goldfinches, consumed few aquatic arthropods, even in spring, late summer, and autumn in riparian areas in our study area (Liu, 2015; Wesner et al., 2020). Also, because goldfinches eat mostly seeds all year, aquatic resources probably aren't very important dietary items. Both species showed similar seasonal trends in PUFA metabolites, suggesting that differences in availability of aquatic resources are likely not the explanatory factor for the seasonal differences documented in this study. The potential impacts of these seasonal differences in omega-3 and omega-6 FAs on thermogenic performance

are uncertain. Highly unsaturated omega-3 FAs may positively impact breeding and flight performance in birds (Pierce and McWilliams, 2014; Martinez del Rio and McWilliams, 2016; Twining et al., 2018). Omega-6 FAs may also be associated with improved exercise metabolic performance in endotherms (Ayre and Hulbert, 1997; Pierce et al., 2005; Price et al., 2011; Pierce and McWilliams, 2014). Thus, lower omega-3, but higher omega-6, fatty acid levels in winter may have contrasting effects on thermogenic performance, but demonstration of such effects will require further study.

Interpreting the direction of seasonal variation in flux through metabolic pathways from single measurements of metabolite levels is difficult, but seasonal changes in metabolite levels do suggest alterations in flux through metabolic pathways. The abundances of complex long-chain lipids (e.g., 1,2-dipalmitoyl-GPC) tended to be lower in winter samples, but winter increases in several acylcarnitines (e.g., behenoylcarnitine, erucoylcarnitine) also occurred. Complex long-chain FAs might be reduced to shorter-chain FAs by peroxisomes (Poulos, 1995; Carter et al., 2019) for delivery to mitochondria, so we hypothesize that winter decreases in long-chain FAs might reflect increases in production of short-chain FAs for delivery to mitochondria for beta-oxidation. Winter increases in several acylcarnitines might be consistent with the increased production of shorter-chain FAs from complex long-chain FAs prior to transport into mitochondria, which could suggest increased demand for use to support beta-oxidation. Increases in beta-oxidation capacity are a common element of the winter phenotype in birds (Marsh and Dawson, 1989; Swanson, 2010). Such increases in fatty acid availability might support shifts

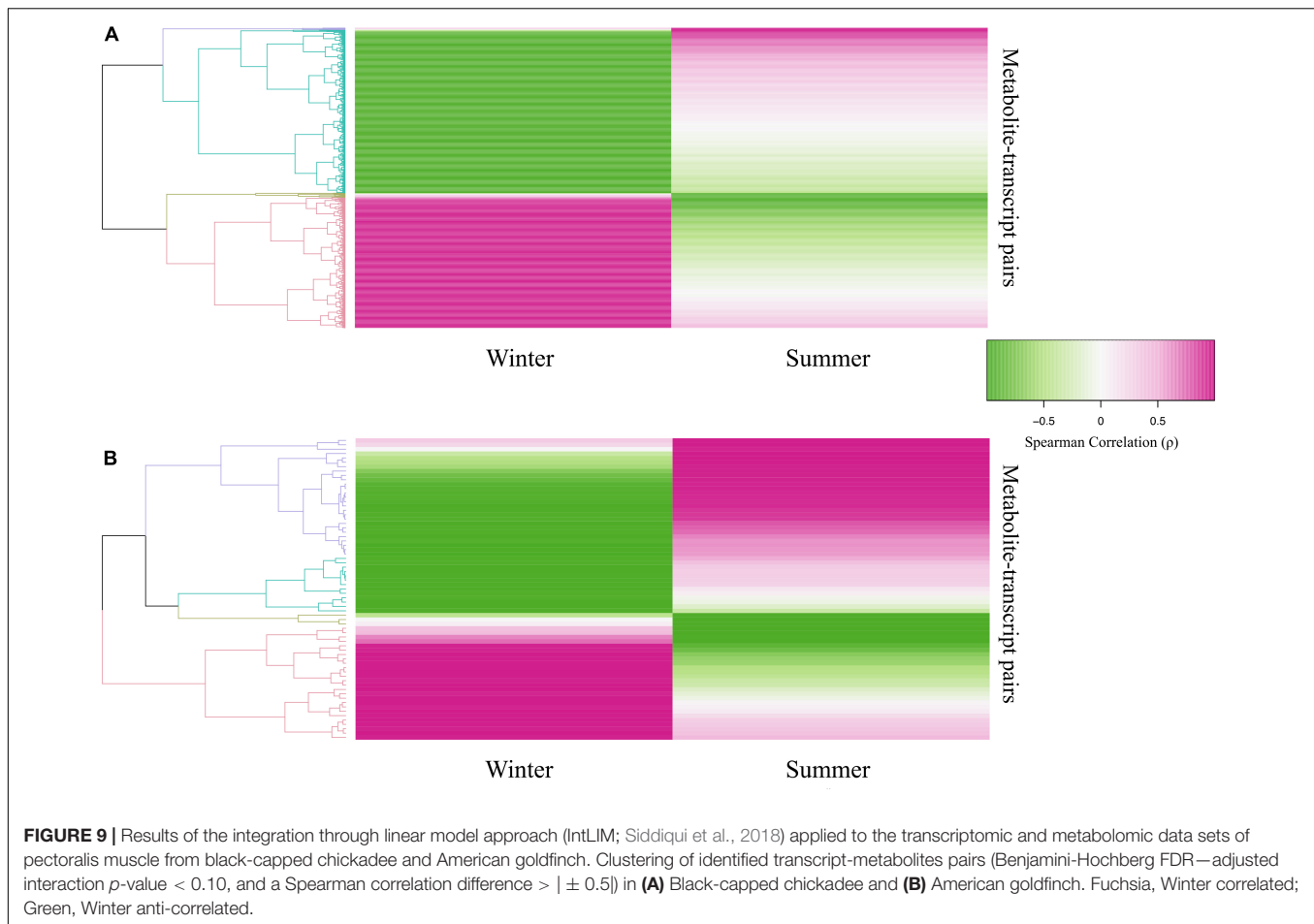


in energy use toward lipid substrates in winter (Marsh and Dawson, 1989; Swanson, 2010). Moreover, the ketone body 3-hydroxybutyrate (BHBA) was elevated in Winter in chickadees but showed a trend toward a winter decrease for goldfinches. The abundance of other ketone bodies also showed winter decreases for goldfinches, which could reflect increased energy demand or subtle changes in substrate availability, perhaps due to a higher proportion of seeds in the winter diet of goldfinches than chickadees. Further studies assessing plasma and/or liver function could shed additional light on seasonal changes in lipid metabolism in these two small bird species.

The reduced abundances of many amino acids in both species from Winter samples suggest potential changes in protein turnover rates in winter birds associated with their higher metabolic rates (Cooper and Swanson, 1994; Liknes et al., 2002). We hypothesize that the generally lower amino acid levels might be associated with increased protein turnover rates in winter birds. Consistent with this hypothesis, cold exposure increased carbon turnover rates in pectoralis muscle of zebra finches (*Taeniopygia guttata*) relative to flight exercise-trained and control birds that were within their thermal neutral

zone (Bauchinger et al., 2010). In addition, exercise-training in human subjects led to higher metabolic rates and higher rates of protein turnover, including both catabolism and synthesis (Rodriguez et al., 2007). Thus, our data showing generally reduced abundances of amino acids in Winter of both species could potentially be consistent with higher winter protein turnover. Further study focusing on seasonal measurement of protein turnover, however, is required to definitively determine how amino acid levels in our study might be related to protein turnover. Moreover, if protein turnover varies between seasons, as we hypothesize, how such variation in protein turnover relates specifically to general winter increases in muscle mass in winter for small birds in cold climates (Swanson, 2010) is uncertain and will also require further study.

Integration of the metabolomic and transcriptomic data not only corroborated the patterns observed from metabolic profiling of pectoralis muscle but helped identify transcripts and pathways whose expression or function might be coordinately regulated in the winter-adaptive phenotype. For example, in chickadees, integrated pathway analyses of significant transcript-metabolite pairs indicated that the Winter correlated



cluster was overrepresented with pathways of amino acid and metabolism of carbohydrates, amino acid biosynthesis, fatty acid and beta-oxidation, nicotinate and nicotinamide metabolism (**Supplementary Table 5**), whereas the Winter anti-correlated cluster was overrepresented with pathways of nucleotide and amino acid derivatives metabolism, lipid metabolism, and serotonin signaling (**Supplementary Tables 6, 9**). Interestingly for goldfinches, integrated pathway analyses highlighted that the Winter correlated cluster was overrepresented mainly with pathways of sphingolipid metabolism, nicotinate and nicotinamide metabolism (**Supplementary Table 7**), whereas the Winter anti-correlated cluster was overrepresented with pathways of lipid metabolism (**Supplementary Tables 8, 10**). Collectively, the overrepresented pathways explained the reduced levels of amino acids, TCA cycle intermediates, and glycolytic intermediates observed in the metabolic profiling of the two species. Most importantly, our combined metabolomic and transcriptomic analyses revealed other key components of the winter-adaptive response in these two species. Specifically, the Winter correlated cluster was overrepresented with pathways of nicotinate and nicotinamide metabolism and degradation; fatty acid and carnitine derivatives biosynthesis; and ornithine, spermidine, and spermine biosynthesis (**Supplementary Table 5**), whereas the Winter anti-correlated cluster was

enriched for nicotinate nicotinamide metabolism, and, ornithine biosynthesis and metabolism, spermidine and spermine biosynthesis in chickadee. As noted earlier for goldfinches, the Winter correlated cluster was overrepresented mainly with pathways of sphingolipid metabolism, and nicotinate and nicotinamide metabolism. Collectively, these results suggest that both chickadees and goldfinches not only ramp up their metabolism of lipids, but also the biosynthesis and metabolism of nicotinamide and carnitine derivatives. Indeed, elevated levels of nicotinamide adenine dinucleotide (NADH) and nicotinamide riboside were observed in Winter for both species, with NADH levels significantly higher in Winter for both species (**Figure 9**). The metabolism of nicotinate and nicotinamide is generally increased in various tissues under conditions associated with elevated metabolic rates in vertebrates. For example, increased temperatures promoted higher growth rates in juvenile sea bass, *Dicentrarchus labrax*, which resulted in an increased metabolism of nicotinate and nicotinamide derivatives (Zhou et al., 2021). This type of metabolism was also increased during growth in fast-growing chicken (*Gallus gallus*) strains compared to slow-growing strains (Willson et al., 2018). Moreover, the metabolism of nicotinate and nicotinamide derivatives was significantly decreased in kidneys of old mice compared to young mice (Ahn et al., 2021). Dietary supplementation of nicotinamide riboside (an NAD⁺

precursor) improved mitochondrial function and rejuvenated muscle stem cells in aged mice (Zhang et al., 2016). These data suggest a general pattern of positive association between metabolism of nicotinate and nicotinamide derivatives and organismal metabolic capacities in vertebrates. Our data showed the abundances of nicotinate and nicotinamide derivatives increased during Winter in pectoralis muscle of both goldfinches and chickadees, and together with the reported elevation of metabolic rates during winter in these species (Cooper and Swanson, 1994; Liknes et al., 2002), our data are consistent with a positive relationship between nicotinate and nicotinamide metabolism and organismal metabolic capacity.

The metabolic profiling together with the integrated metabolomic and transcriptomic analyses revealed key components of the winter-adaptive response common to both black-capped chickadees and American goldfinches. During winter, the metabolism of lipids is increased with a concomitant increase of nicotinate and nicotinamide metabolism to increase the abundance of NAD^+/NADH . Higher levels of NADH and nicotinamide riboside (Figure 8) likely are associated with increases in thermogenic capacity of their pectoralis muscle, the primary thermogenic organ in small birds. Nicotinamide adenine dinucleotide (NAD^+) and its reduced form NADH are important molecules involved in energy production, function, regeneration, adaption to exercise, aging, stress response, and adaptation to environmental changes in muscles (Li et al., 2009; Goody and Henry, 2018; Xie et al., 2020; White and Schenk, 2022). In addition to the widely recognized roles of NAD^+ as a coenzyme in energy metabolism, we hypothesize that it also serves to increase metabolic output during winter to increase thermogenic capacity in small birds *via* increased metabolism of lipids, which would require higher amounts of NAD^+/NADH (Lutkewitte et al., 2019; Yamaguchi et al., 2019). Although the increased metabolic output of small passerines living in regions with seasonal temperature fluctuations are well documented (Swanson and Vézina, 2015; Li et al., 2017; Wang et al., 2019), the mechanisms that underpin this increase are not fully understood. Our results suggest that increased levels of NAD^+/NADH and their derivatives are a potential mechanism to facilitate the increased metabolic output of the pectoralis muscle for thermogenesis during winter. Although mammalian cells can generate NAD^+ *de novo* from dietary Trp *via* the kynurenine pathway or from the Preiss–Handler pathway from nicotinate (Goody and Henry, 2018; Xie et al., 2020), we are not aware if avian cells can also perform these pathways. However, we suspect that avian cells can generate NAD^+ *de novo* from dietary Trp as the abundance of kynurenine, an intermediate of Trp metabolism (Martin et al., 2020) is lower during Winter for both species, significantly so for chickadees (Figure 7). Similar to mammalian cells, the salvage pathway is the likely primary route given that its precursor, nicotinamide riboside levels are elevated during Winter for both species, where levels were significantly higher in chickadees (Figure 8). Although decreases in amino acid abundances might reflect decreased dietary protein intake during winter, another possible explanation is that amino acids are being shuttled, as precursors to maintain a relatively higher level of nicotinate and nicotinamide metabolism. Notably, transport of

small molecules and metabolism of amino acids were among the most overrepresented pathways in both species, coincident with the decline in the abundance of amino acids as indicated by the metabolic profiling [Figures 3A, 5; Li et al. (2009)]. Future studies should address a causal role for upregulation of the nicotinate and nicotinamide metabolic pathway in promoting elevated winter thermogenic capacities in small birds.

Our major findings regarding the metabolic changes during winter for both black-capped chickadees and American goldfinches can be summarized as a model with four main hypotheses: (1) Metabolism of lipids is the apparent main source of energy during winter; (2) reduced amino acid levels in winter are consistent with seasonal changes in protein turnover that might contribute to seasonal variation in muscle mass; (3) accumulated NADH and nicotinamide riboside should facilitate the increased metabolic output needed for thermogenesis during winter; and (4) increased biosynthesis and metabolism of nicotinates and nicotinamides is a potential mechanistic adaption underpinning seasonal metabolic flexibility. These hypotheses offer new plausible directions for further research into the mechanism of the winter-adaptive response in small passerine birds.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of South Dakota Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BW and DS designed, conducted the research, and wrote the manuscript. BW analyzed the data and integrated the transcriptomic and metabolomic data. Both authors reviewed, edited, and approved the final manuscript.

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Manipulation of Heat Dissipation Capacity Affects Avian Reproductive Performance and Output

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Animal life requires hard work but the ability to endure such workload appears to be limited. Heat dissipation limit (HDL) hypothesis proposes that the capacity to dissipate the excess of body heat during hard work may limit sustained energy use. Experimental facilitations of heat loss rate via feather-clipping in free-living birds seem to support HDL hypothesis but testing of HDL through laboratory experiments under controlled conditions is not reported. We employed a two-factorial experimental design to test HDL hypothesis by manipulating the capacity to dissipate heat through exposure of captive zebra finches (*Taeniopygia guttata*) to a cold and warm ambient temperature (14°C and 25°C), and through manipulation of the insulating layer of feathers around the brood patch in females (clipped and unclipped). To simulate foraging costs encountered in the wild we induced foraging effort by employing a feeding system that necessitated hovering to access food, which increased energetic costs of reproduction despite *ad libitum* conditions in captivity. We quantified the outcome of reproductive performance at the level of both parents, females, and offspring. Thermal limitations due to warm temperature appeared at the beginning of reproduction for both parents with lower egg-laying success, smaller clutch size and lower egg mass, compared to the cold. After hatching, females with an enhanced ability to dissipate heat through feather-clipping revealed higher body mass compared to unclipped females, and these clipped females also raised heavier and bigger nestlings. Higher levels for oxidative stress in plasma of females were detected prior to reproduction in warm conditions than in the cold. However, oxidative stress biomarkers of mothers were neither affected by temperature nor by feather-clipping during the reproductive activities. We document upregulation of antioxidant capacity during reproduction that seems to prevent increased levels of oxidative stress possibly due to the cost of female body condition and offspring growth. Our study on reproduction under laboratory-controlled conditions corroborates evidence in line with the HDL hypothesis. The link between temperature-constrained sustained performance and reproductive output in terms of quality and quantity is of particular interest in light of the current climate change, and illustrates the emerging risks to avian populations.

Keywords: heat dissipation limit, reproduction, oxidative stress, sustained energy, nestling provisioning, laboratory avian model, temperature manipulation

INTRODUCTION

Energy is fundamental for life, its availability and use determines the reproductive success and survival of organisms (Carey, 1996). Along the annual cycle, animals often face challenging conditions requiring particularly high levels of energy. During reproduction for instance, adults spend an increased amount of energy for a sustained period to establish a territory, find a mate, build a nest, lay and incubate eggs, and finally raise their offspring (Drent and Daan, 1980). While it is generally accepted that the rate at which animals can expend energy is limited we lack detailed understanding of such limitations (Drent and Daan, 1980; Peterson et al., 1990; Weiner, 1992; Hammond and Diamond, 1997; Speakman and Król, 2010a). Several hypotheses suggest that energy limitations can be imposed by either external (e.g., food availability; Speakman et al., 2003) or internal physiological and morphological factors (Drent and Daan, 1980; Hammond and Diamond, 1997; West et al., 1999). For instance, the capacity to process and digest the incoming energy (“central limitation hypothesis,” Drent and Daan, 1980; Koteja, 1996; Thurber et al., 2019), to deliver nutrients to organs and tissues limits energy (“metabolic theory of ecology,” West et al., 1999), or even the working capacity of the organs themselves (“peripheral limitation hypothesis,” Hammond and Diamond, 1997), may constrain maximum and sustained energy expenditure. Lately, it has been proposed that the capacity to dissipate the excess of body heat may limit the use of energy, forwarded as the heat dissipation limit (HDL) hypothesis (Król et al., 2007; Speakman and Król, 2010b).

First observations of cold exposure as experimental manipulation that enhances the rate heat is dissipated from the body revealed that the capacity to thermoregulate in order to avoid overheating may constrain reproductive performance and thus optimization of energy use (Johnson and Speakman, 2001; Król and Speakman, 2003). When exposed to a cold ambient temperature of 8°C during lactation (i.e., the most energetic demanding phase) mice (outbred MF1, *Mus musculus*) increased food intake, produced more milk and raised heavier offspring compared to mice exposed to a higher ambient temperature of 21°C (Johnson and Speakman, 2001; Król et al., 2007). Avian heat production during reproduction seems less directly affected as in mammals during lactation, but chick-rearing requires an intensive physical workload for food provisioning. Zebra finches (*Taeniopygia guttata*) raised offspring heavier in mass and a more advanced in structural size (tarsus length at day 28 post hatching) when exposed to a cold temperature of 14°C than those, exposed to 30°C (Andrew et al., 2017) a temperature considered within the thermoneutral zone (TNZ in zebra finches varies from 30°C to 38°C; Calder, 1964). Further studies have provided support for HDL hypothesis by manipulating and enhancing heat dissipation via decrease of insulation, either through fur removal in mammals (Król et al., 2007), or feather-clipping in birds (Nilsson and Nord, 2018). Increased ability to dissipate heat via a feather-clipping in female blue tits (*Cyanistes caeruleus*) seemed to have relaxed energetic limitations compared to un-manipulated control birds (Nilsson and Nord, 2018); feather-clipped females could minimize body mass loss compared to

controls and also raised heavier nestlings (Nilsson and Nord, 2018). Feather-clipped tree swallows (*Tachycineta bicolor*) raised nestlings with higher body mass and maintained higher workload and lower day-time body temperature, compared to controls (Tapper et al., 2020a). Those effects of ambient temperature and treatment with increased heat loss rate in both mammals and birds seem to be consistent with HDL hypothesis in endotherms (although see Thurber et al., 2019 for humans, Rogowitz, 1998; Yang et al., 2013), resulting in high reproductive performance and output. So far, evidence supporting HDL hypothesis in birds exists only under field conditions while laboratory studies in birds are yet to be implemented. Understanding the costs of oxidative stress associated with thermal limitations, as well as the costs associated with avoiding oxidative stress during periods of high workload, is also required.

Aerobic metabolism results in reactive oxygen species (ROS) production, and any imbalance between ROS and the antioxidant defense mechanism toward ROS may come with a consequence of oxidative stress damaging biomolecules (Sies, 1997; Finkel and Holbrook, 2000; Alonso-Alvarez et al., 2006; Rahal et al., 2014). However, organisms may employ different ways to maintain oxidative balance. The first line of protection tries to minimize ROS production within cells either through membrane composition or uncoupling of oxygen consumption and ATP production (Monaghan et al., 2009; Alan and McWilliams, 2013). Antioxidant defense system comes as a second line with antioxidants to counteract ROS and plays a significant role in protection against oxidative stress (Halliwell and Gutteridge, 1999; Garratt et al., 2011; Speakman and Selman, 2011; Yang et al., 2013; Brzęk et al., 2014). Antioxidants may occur in enzymatic and non-enzymatic form in the intracellular and extracellular environment (Nimse and Pal, 2015). Maintaining oxidative balance might be a challenge when organisms face metabolic changes due to exposure to low or high ambient temperatures or increased physical activities during reproduction. For instance, current evidence shows that breeding adults are rather confronted with a reduced amount of antioxidant capacity (Wiersma et al., 2004; Losdat et al., 2011; van de Crommenacker et al., 2012; Costantini et al., 2014a) implying perhaps that breeding adults may not be able to invest enough for self-maintenance (i.e., by self-feeding and hence increasing antioxidant capacity with exogenous antioxidants) or reflecting the antioxidant response to oxidative stress (Costantini, 2011). On the other hand, oxidative damage is not always detected due to elevated reproductive performance in both birds and mammals (Ołdakowski et al., 2012; Brzęk et al., 2014; Costantini et al., 2014b). Exposure to high ambient temperature may also cause a series of physiological costs, such as increased body temperature and metabolic rate and oxidative stress (Lin et al., 2006; Mestre-Alfaro et al., 2012; Khan et al., 2021). Given the suite of benefits of experimentally enhancing the excess of body heat during reproduction, it is reasonable to suggest simultaneous benefits for self-maintenance investing toward current reproduction. To the best of our knowledge, there are only a few studies investigating the consequences of ambient temperature in oxidative stress during reproduction (lactating Mongolian gerbils; Yang et al., 2013, see review in broiler chickens; Khan et al., 2021), and

no study has been conducted until now to investigate the benefits or costs in terms of oxidative stress under experimental facilitations of the capacity to dissipate heat in endotherms during hard work.

To test HDL hypothesis, we employed two separate manipulations of the capacity to dissipate heat in captive zebra finches (*Taeniopygia guttata*). In a two-factorial experimental design, we manipulated the capacity to dissipate heat through exposure of zebra finches to cold and a warm ambient temperature (factor I, 14°C and 25°C respectively; established 1 month before hatching), and through manipulation of the insulating layer of feathers around the brood patch (factor II, feather-clipped versus unclipped; applied on day 6 post-hatching). Initially, we estimated the effects of ambient temperature on both parents by following egg-laying and egg production. Female reproductive performance was determined by measuring body mass and food intake during offspring provisioning. Additional maternal measurements of several oxidative stress biomarkers were implemented to increase our knowledge on the potential costs or benefits when removing the problem of heat dissipation. Reproductive output was estimated by measuring body mass of the nestlings several times along their development, tarsus (an indicator of skeletal development) and wing length (a predictor for bone development and feather growth) when nestlings reached almost a fully juvenile size (35 days old). If indeed the metabolic performance is limited by the capacity to dissipate body heat, we formulated several predictions: (a) feather-clipped females will show higher body mass and food intake than unclipped females, (b) the enhanced ability to dissipate heat will enable females to invest more in self-maintenance resulting in lower oxidative stress than the unclipped controls, and this effect will be more pronounced in warm compared to cold conditions, (c) females with increased heat loss rate (either through cold exposure or feather-clip treatment) will raise heavier and more developed nestlings than the unclipped control females.

MATERIALS AND METHODS

Individuals and Temperature Set-Up

For this study, we applied two separate manipulations of the capacity to dissipate heat using captive zebra finches (*Taeniopygia guttata*) in a two-factorial experimental design. Temperature manipulations influence the capacity to transfer body heat since heat loss depends on the difference between the body and the ambient temperature (Speakman and Król, 2010b), and thus, before reproduction, we exposed total 82 pairs of birds to 14°C and 25°C with 39 and 43 pairs, respectively (factor I). Both temperatures are considered below the thermoneutral zone (TNZ) in zebra finches, which ranges from 30°C to 38°C (Calder, 1964; Briga and Verhulst, 2017). In the breeding season, zebra finches under natural conditions may experience temperatures ranging from 10°C to 30°C (even in some cases above 36°C) but most frequently they lay eggs at ambient temperatures of 18°C to 20°C (Griffith et al., 2017). In laboratory conditions, ambient temperature for breeding zebra finches is set from 18°C to 22°C

(Rutkowska et al., 2005; Williamson et al., 2008; Arct et al., 2010; Olson et al., 2014) for high reproductive success. For this reason, we applied two ambient temperatures with a difference of at least ten degrees, one below 18°C and one above 22°C, considering them as cold and warm, respectively. We monitored egg-laying success and hatching success between the two ambient temperatures. About 82% of the 39 pairs (32 out of 39) laid eggs in the cold conditions, while about 51% of the 43 pairs (22 out of 43) laid eggs in warm conditions. From those pairs that laid eggs, a total of 26 out of 32 chicks hatched in cold conditions and 18 out of 22 in the warm ($\chi^2 = 0.002$, $p = 0.95$). During reproduction, and more specifically on day 6 post-hatching, we applied our second manipulation of heat dissipation via manipulation of the insulating layer of feathers around the brood patch (factor II, feather-clipped versus unclipped females). At the end, 26 out of 26 pairs successfully raised fully developed chicks until independence at the cold conditions (14 feather-clipped, 12 unclipped), while 14 pairs out of 18 raised fully developed chicks at the warm (7 feather-clipped, 7 unclipped) ($\chi^2 = 6.35$, $p = 0.01$).

In each chamber, the ambient temperature was recorded with six data loggers (thermochrons, DS1921H-F5, iButtonLink, Maxim Integrated products, United States) and the humidity with one additional (hygrochron, DS1923-F5, iButtonLink, Maxim Integrated products, United States) to monitor and maintain the ambient temperature and humidity within established levels. All data loggers were synchronized at the same time to record three times when lights were on (“daytime”) and three times when lights off (“nighttime”). Temperature significantly differed between daytime and nighttime for both chambers ($F_{1,7789} = 6.03$, $p < 0.001$). Humidity significantly differed between daytime and nighttime ($F_{1,940} = 46.71$, $p < 0.001$) but it did not differ between the two chambers ($F_{1,940} = 0.001$, $p = 0.97$). The summary of the statistics of temperature and humidity separated for day and night for each chamber is shown in **Supplementary Table 1**.

Acclimation Period

Our laboratory colony of zebra finches is usually habituated in a common outdoor aviary at the Institute of Environmental Sciences, Jagiellonian University, Krakow. Thus, at the beginning of the experiment we moved the experimental birds and separated them into the two indoor climatic chambers. Birds were initially housed in individual cages (70 × 70 × 45 cm; L × W × H) with two birds of the same sex in each cage and visually separated from other birds. We let birds to acclimatize for 2 weeks at the new laboratory conditions with a photoperiod 13:11 (L:D) with lights on at 7 AM and lights off at 8 PM. After 2 weeks of acclimation, we implemented a new feeding system for every cage to increase physical activity and foraging costs for all birds (see details below). The training procedure lasted for 2 months. When all birds were trained to the new feeding system, we left them for 3 weeks at the ambient temperatures of 14°C and 25°C before the mating (see a summary of the experimental timeline in **Supplementary Figure 1**).

For this experiment, we implemented a feeding system for every cage comparable to the “high foraging cost feeders” of

Koetsier and Verhulst (2011). Such high foraging costs feeders may increase foraging effort by increasing time for foraging and the flights for obtaining food as it was previously shown for zebra finches (Koetsier and Verhulst, 2011; Yap et al., 2017), simulating foraging costs in field conditions. Female zebra finches revealed to have increased flight muscle mass, lung mass and heart mass in response to high foraging cost feeders compared to females exposed to regular feeders (although no differences were detected in males, Yap et al., 2017). Therefore, we constructed a similar feeding system using a transparent plastic feeder and opened one hole fitted with a tube. The feeder was mounted 40 cm from the floor of the cage and initially a wooden stick of 15 cm was attached for perching. After 4 days, we gradually shortened the wooden stick size down to 7 cm. Birds continued to have access to food by perching on the wooden stick for a total of 9 days, thereafter which we removed the perch to start training the birds to fly toward the foraging hole and hover briefly to collect the seeds (see **Supplementary Figure 2** and uploaded video at **Supplementary Material**). On the floor of the cage, below the feeder, a plastic container and a covered glass tube collected all the spilled seeds. This prevented the birds to collect any spilled seeds on the floor and the containers were emptied every second day. During the whole experiment and reproduction, all birds had unlimited access to food (a mixture of different millet species; Megan, Poland), but as outlined we did increase their physical exercise for foraging to simulate natural conditions. Birds were trained to the new high foraging cost system for 12 days. For more information of the training procedure and the hovering behavior of the birds, see **Supplementary Material**.

Experimental Procedure

After the training to the new feeding system and 3 additional weeks of acclimation period, we randomly mated the birds with unrelated partners and randomly separated the pairs in four different blocks. Each block was assigned for mating with 4 days of interval. On the mating day, each individual cage was equipped with an internal carton-made nest box and nest-material (wood wool and shredded toilet paper) which was replaced every day until birds stopped using it. After the mating, nest boxes were checked every morning between 10 AM and 11 AM in order to inspect the nest-building stage, the egg laying date (females lay one egg per day early in the morning) and the start of incubation. During the egg-laying period, we marked all eggs alphabetically with a pen, in the sequence they were laid, and each was weighed with an electronic balance (Kern MM 60 2N $\pm$ 0.01, Kern & Sohn GmbH, Germany). When incubation started, birds were supplied three times per week with a small spoon of a mix of chopped hard boiled eggs (with the shell), grated carrot and supplementary vitamins. Birds also had unlimited access to water and to a piece of cuttlebone for the entire period.

Adult Measurements

Just before mating, we caught all birds early in the morning to measure initial body mass using an electronic balance ($\pm$ 0.1 g; KERN 440-45N, Kern & Sohn, GmbH, Germany). When first

eggs hatched, which we assigned as day 0, we also measured female body mass just after lights on, and repeated the body mass measurement on day 4, 6, 8, 10, 12, 16, and day 35 (see also **Supplementary Figures 1, 5**). At the same time that we caught each female from the cage, we also measured nestlings to follow growth rate (more details on nestling measurements see below). On day 4, we removed the male from the cage to increase the physical activity of the female, assuring that the reproductive effort is only operated from a single parent. On day 6, we applied the feather clip manipulation by trimming the feathers around the brood patch and those covering the pectoral muscles. We established two manipulation groups: feather-clipped and unclipped females, which were handled and measured in the same way but not feather-clipped (sham-manipulation). On day 12, just before lights off, we captured the females from inside the nest-box and we performed respiratory measurements to estimate thermal conductance of the females (for more details see **Supplementary Material**). Feather-clipped females revealed significantly higher thermal conductance, hence heat loss (up to 9.5% increase), than the unclipped ones (**Supplementary Figure 4**). Last, females were blood-sampled three times during the experiment: (a) just before mating (after taking initial body mass), (b) on day 13 just after respiratory measurements, and (c) at the end of the experiment, on day 35, when chicks reached full independence.

Nestlings

During the expected hatching date, we monitored the nests every day between 10 AM and 11 AM to inspect for possible newly fresh hatchlings. We distinguished day of hatching based on the presence of eggshells' leftovers and the appearance of the hatchlings, since fresh hatchlings are reddish and wet. The true hatching date was recorded as day 0 and the true age of each hatchling was assigned accordingly. We immediately weighed each hatchling with an electronic balance (Kern MM 60 2N $\pm$ 0.01, Kern & Sohn GmbH, Germany), marked them by nail clipping their claw and returned them to the nest. Body mass of the nestlings was measured several times during mornings (from 7 AM to 10 AM) until they reached adult size to capture growth rate (day 0, 4, 6, 8, 10, 12, 16, and day 35, see also **Supplementary Figure 5**). On day 16, just before fledgling, we also ringed chicks with an individually numbered aluminum ring and measured tarsus and wing length. As a part of a different study, we collected blood sample from all nestlings on day 16. Nestling survival was followed up to 35 days of age, which was the last time we measured their body mass, tarsus and wing length to estimate their final adult size.

Food Intake

We estimated food intake by measuring the initial seed mass and after 48-h period the final seed mass. For the food intake we also took into account the spilled seeds in the container that were not obtained and consumed from the birds. We measured food intake over a 48-h period during the peak of food provisioning and reproductive performance on day 4 to 6, day 6 to 8, day 8 to 10, and day 10 to 12.

Blood Sampling and Oxidative Stress Biomarkers

We blood-sampled females three times: (a) before mating, (b) on day 13, just after respiratory measurements, and (c) at the end of the experiment, on day 35, when chicks reached full independence. Blood sampling always took part early in the morning just after lights on. After a brachial vein puncture, we collected blood sample of 75 μ l in capillaries and stored in heparinized Eppendorfs 100 μ l. Blood sample was immediately centrifuged for 10 min at 3340 g to separate plasma (Centrifuge MPW-56, MPW Med. instruments). Plasma sample was stored at -80°C until further analyses which took place within 1 month after the end of the experiment. Following the protocol of Costantini and Dell’Omo (2006) for the d-ROM test, we estimated oxidative damage and the results are expressed as mmol of H_2O_2 equivalents. The oxy-adsorbent (OXY) test measures the total non-enzymatic antioxidant capacity (Costantini and Dell’Omo, 2006) by quantifying the ability of plasmatic antioxidants to cope with hypochlorous acid (HClO); an endogenously produced oxidant. We followed the protocol of Sudyka et al. (2016) and values are expressed as mmol of HClO neutralized. The plasma concentration of uric acid was measured by the endpoint uric acid assay kit (Diacron International, Grosseto, Italy) and we followed the protocol of Zagkle et al. (2020). All analyses for the colorimetric assays were run in duplicate using an absorbance reader (Sunrise, Tecan’s Magellan, Tecan Trading, Switzerland). In the end, we estimated oxidative stress index by calculating the ratio of the oxidative damage to the total non-enzymatic antioxidant capacity in the plasma multiplied by 1000.

Statistical Analysis

We performed all analysis using R computer software (R version 4.1.2, R Core Team, 2021). For the linear mixed effect models we used *lme4* package (Bates et al., 2015) and *lmerTest* package (Kuznetsova et al., 2017) to calculate the degrees of freedom and p values. *Post hoc* comparisons were performed using Tukey method in case of two groups of means, and Sidak method for multiple groups of means. Least square means (LSM) and standard error means ($\pm$ SE) were calculated using *emmeans* package (Lenth, 2022). All non-significant interactions ($p > 0.05$) were removed from the models. We checked all our models for normality assumptions and homogeneity of variance by visual inspection of the residuals and in some cases variables (i.e., clutch size, uric acid and oxidative stress index) were log-transformed to meet the assumptions of normality. All figures presented are based on raw data. The sample size in many biological experimental studies may not always reach a sufficient amount (i.e., for logistic reasons as in this study) and thus decreasing considerably the statistical power. We, therefore, present and interpret the biological effects in a language of evidence (Muff et al., 2022) and clarity (Dushoff et al., 2019) rather than in the cut-off binary decision of the p -Value < 0.05 .

Egg Mass

We tested differences in egg mass by performing linear mixed effect model analysis, with egg mass the response variable and the ambient temperature as the predictor. Clutch size (the number of eggs) and the body mass of the mother were included as covariates, and the identity of the nest box was included as a random effect to control for any dependence laid on the same nest by the same mother.

Female Body Mass

We performed two-way ANOVA for testing differences in body mass in mothers at the beginning of the experiment between the ambient temperatures. Linear mixed-effect model analysis was performed for body mass in females including ambient temperature and nestling age (day 0, 4, and 6 - which was the day of feather-clip manipulation) as fixed effects and their interaction. Another linear mixed effect model was performed for analysis of female body mass with the explanatory variables of ambient temperature (factor I; cold and warm), feather-clip manipulation (factor II; feather-clipped, unclipped), the nestling age (day 8, 10, 12, 16, and 35) and their interaction. Number of chicks was included as a covariate. For both models, the identity of the female was included as a random effect.

Oxidative Status

We tested for differences in oxidative stress biomarkers (response variable) in females between the two ambient temperatures (factor I) performing one-way ANOVA when sampled just before reproduction. We sampled mothers at the nesting age of 13 and 35 days old, at the peak of food provisioning and toward the end of offspring rearing respectively. The given oxidative stress biomarker was set as a response variable and we added nestling age (categorical variable with two levels: 13 and 35), ambient temperature and the feather-clip manipulation groups as predictors and their interaction as well. The body mass of the female was included as a covariate, while the identification number of the female was a random effect to account for the repeated measures.

Food Intake

We tested for differences in food intake between the two ambient temperature groups before the feather-clip manipulation, at the nestling age of 4 to 6 days old performing ANCOVA. We included female body mass and clutch size as covariates. After feather-clip manipulation, we performed a linear mixed effect model to detect variation in food intake during the chick development over three 48 h periods; (1) from 6 to 8 days old of chicks, (2) from 8 to 10 days old, and (3) from 10 to 12 days old. As predictor variables, we added ambient temperature, feather-clip manipulation, the sampling point, and their interaction. Brood size and female body mass were included in the model as covariates.

Nestling Development

(a) To test differences in body mass, tarsus, and wing length (response variables) between the two ambient temperatures (factor I) until the feather-clip manipulation that took place on day 6 we performed linear mixed effect model including

interaction between ambient temperature and experiment day. Brood size was also included as a covariate to control for any differences between small and large broods and the identity of the female and the identity of the chicks were set as random effects. (b) We performed linear models over the nestling age since the relationship appeared to be linear (contrary to the asymptomatic growth slope) to test differences in body mass, wing, and tarsus length in nestlings (response variable) between the two ambient temperatures (factor I) and the feather-clip manipulation groups (factor II). For this model we included a third factor of the experimental day (factor III; levels: day 8, 10, 12, 16, and 35) and the interaction between the three factors (factor I $\times$ factor II $\times$ factor III). The brood size was included as a covariate to control for any differences in nestling development between small and large broods. The identity of the chicks was included as a random effect to account for the repeated measurements during the development nested in the identity of the mother to control for any dependence in the nestling development from the same mothers.

RESULTS

Biparental Traits

A total of 82 pairs were exposed to either cold conditions of 14°C ($n = 39$) or warm conditions of 25°C ($n = 43$). Birds were more successful in laying eggs at the cold compared to warm conditions; about 82% of the 39 pairs (32 out of 39) in the cold and about 51% of the 43 pairs (22 out of 43) in the warm laid eggs ($\chi^2 = 8.67$, $p = 0.003$; **Figure 1A**). From those pairs that laid eggs, a total of 26 out of 32 chicks hatched in the cold, and 18 out of 22 in the warm. There was no evidence that ambient temperature influenced hatching success ($\chi^2 = 0.002$, $p = 0.95$). When chicks hatched (day 0), there was moderate evidence in clutch size (after log-transformation) between the two ambient conditions ($F_{1,52} = 5.7$, $p = 0.02$; one-way ANOVA, **Figure 1B**); females in the warm laid significantly smaller clutches than the females in the cold. Variation in egg mass (ANCOVA) was explained by the ambient temperature ($F_{1,49.5} = 5.7$, $p = 0.02$; **Figure 1C**) and female body mass ($t = 2.52$, $F_{1,46.7} = 6.3$, $p = 0.01$), while there was no evidence that the clutch size had an effect on egg mass ($t = 0.91$, $F_{1,37.9} = 0.8$, $p = 0.36$).

Female Traits

Body Mass

Initial body mass measured before mating did not differ between the two ambient temperatures ($F_{1,81} = 2.28$, $p = 0.13$; one-way ANOVA). Body mass decreased continuously from the day that chicks hatched until day 6 ($F_{2,78.6} = 297.8$, $p < 0.001$), but no difference was found between the two ambient temperatures ($F_{1,45.5} = 2.72$, $p = 0.11$). Interaction between ambient temperature and nestling age was found to be statistically unclear. We applied the feather-clip manipulation when chicks were 6 days old and there was no evidence that the body mass of the females differ between the experimental groups ($F_{1,38} = 2.02$, $p = 0.16$). Linear mixed effect model analysis revealed that there was strong evidence that nestling

age was associated with female body mass ($F_{4,157.0} = 44.4$, $p < 0.001$); body mass decreased significantly for all females during nestling-rearing period. We found weak evidence for an interactive effect between feather-clip manipulation and ambient temperature ($F_{1,36.9} = 3.7$, $p = 0.06$). Unclipped females in the warm temperature revealed the lowest body mass (11.9 ± 0.4 ; LSM $\pm$ SE) compared to the feather-clipped females in the warm (13.2 ± 0.4 ; LSM $\pm$ SE), unclipped (13 ± 0.3 ; LSM $\pm$ SE) and feather-clipped (12.9 ± 0.3 ; LSM $\pm$ SE) in the cold (model results in **Supplementary Table 2** and *post hoc* analysis in **Supplementary Table 3**). We also performed ANCOVA for female body mass (g) on day 16 and found moderate evidence for an interactive effect between ambient temperature and feather-clip manipulation ($F_{1,37} = 4.26$, $p = 0.04$). Unclipped mothers in the warm temperature revealed the lowest body mass in comparison to the other three groups (**Figure 2A**).

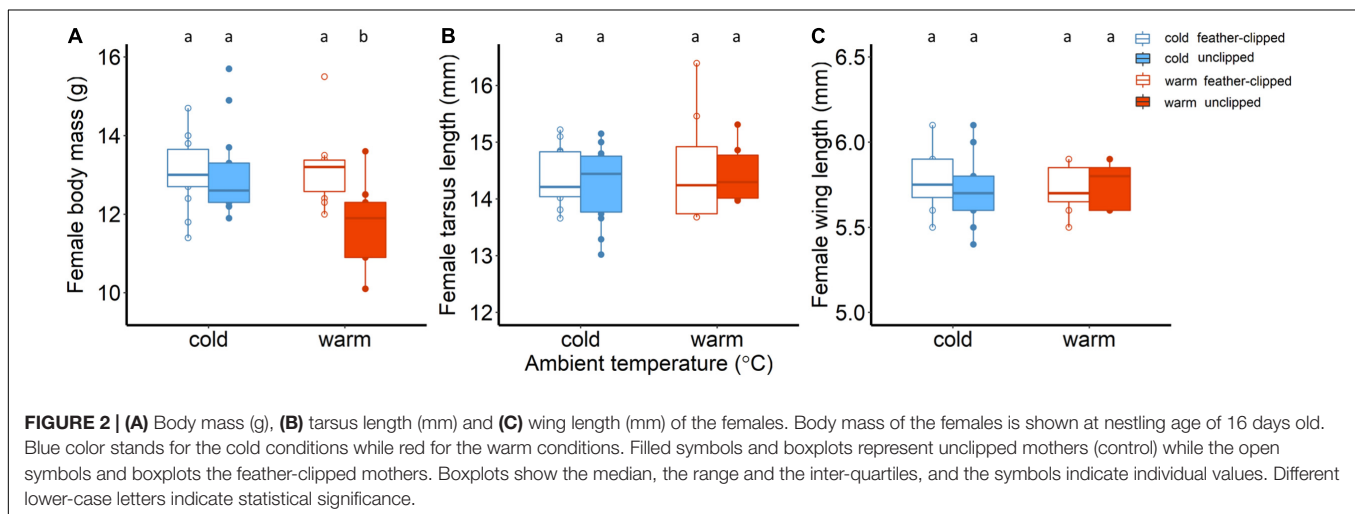
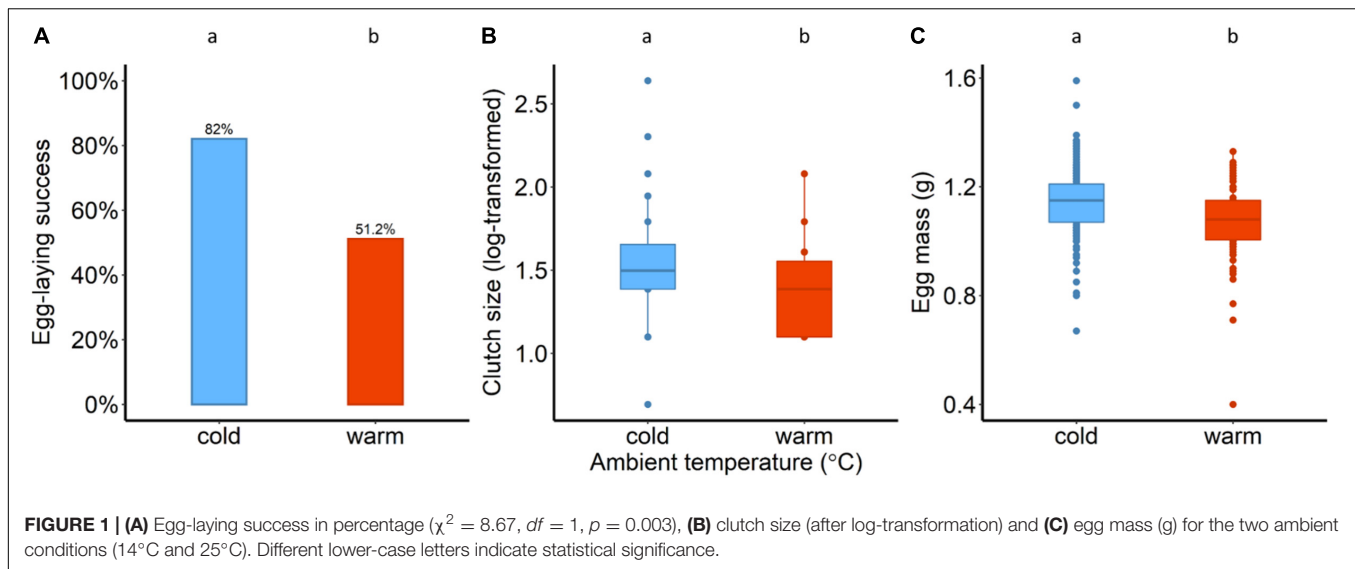
Oxidative – Antioxidant Status

Effect of Temperature Before Reproduction

There was no evidence for an influence of ambient temperature on uric acid when birds were sampled in the morning, just before mating ($F_{1,72} = 0.61$, $p = 0.43$; **Figure 3A**). Similarly, no evidence of ambient temperature in non-enzymatic antioxidant capacity ($F_{1,69} = 0.25$, $p = 0.61$; **Figure 3B**). We found moderate evidence in oxidative damage, measured as d-ROM, between the two groups of ambient temperature ($F_{1,73} = 5.36$, $p = 0.02$); females in the warm conditions revealed higher oxidative damage (LSM $\pm$ SE: 1.75 ± 0.07) than females in the cold conditions (LSM $\pm$ SE: 1.49 ± 0.07) (**Figure 3C**). Differences between the two ambient temperature in oxidative stress index (after log-transformation), calculated as the ratio of the oxidative damage to the total non-enzymatic antioxidant capacity multiplied by 1000 (Costantini et al., 2007), were not statistically clear ($F_{1,68} = 2.46$, $p = 0.12$; **Figure 3D**).

Effect of Temperature and Feather-Clipping During Offspring Rearing Period

We found no evidence for an interactive effect between sample point, feather-clipping and ambient temperature in any of the oxidative stress biomarkers and thus removed these from the models. There was a statistical clear difference in uric acid (after log-transformation) between the two sampling points ($F_{1,38.6} = 65.5$, $p < 0.001$); uric acid in females was higher at the end of the offspring rearing period (LSM $\pm$ SE: -0.05 ± 0.07 , on day 35) compared to the peak of offspring rearing period (LSM $\pm$ SE: -0.76 ± 0.07 , on day 13). While ambient temperature did not explain any variation in uric acid ($F_{1,38.2} = 2.9$, $p = 0.09$), there was moderate evidence that feather-clip manipulation had an effect in uric acid ($F_{1,39.0} = 4.6$, $p = 0.03$). Feather-clipped females regardless of the ambient temperature revealed higher uric acid (LSM $\pm$ SE: -0.28 ± 0.08) than the un-manipulated (LSM $\pm$ SE: -0.53 ± 0.08) females (**Figure 3A**). Non-enzymatic antioxidant capacity also differed significantly between the two sampling points ($F_{1,36.5} = 20.23$, $p < 0.001$); non-enzymatic antioxidant capacity was significantly lower (LSM $\pm$ SE: 74.7 ± 5.5) in females toward the end of

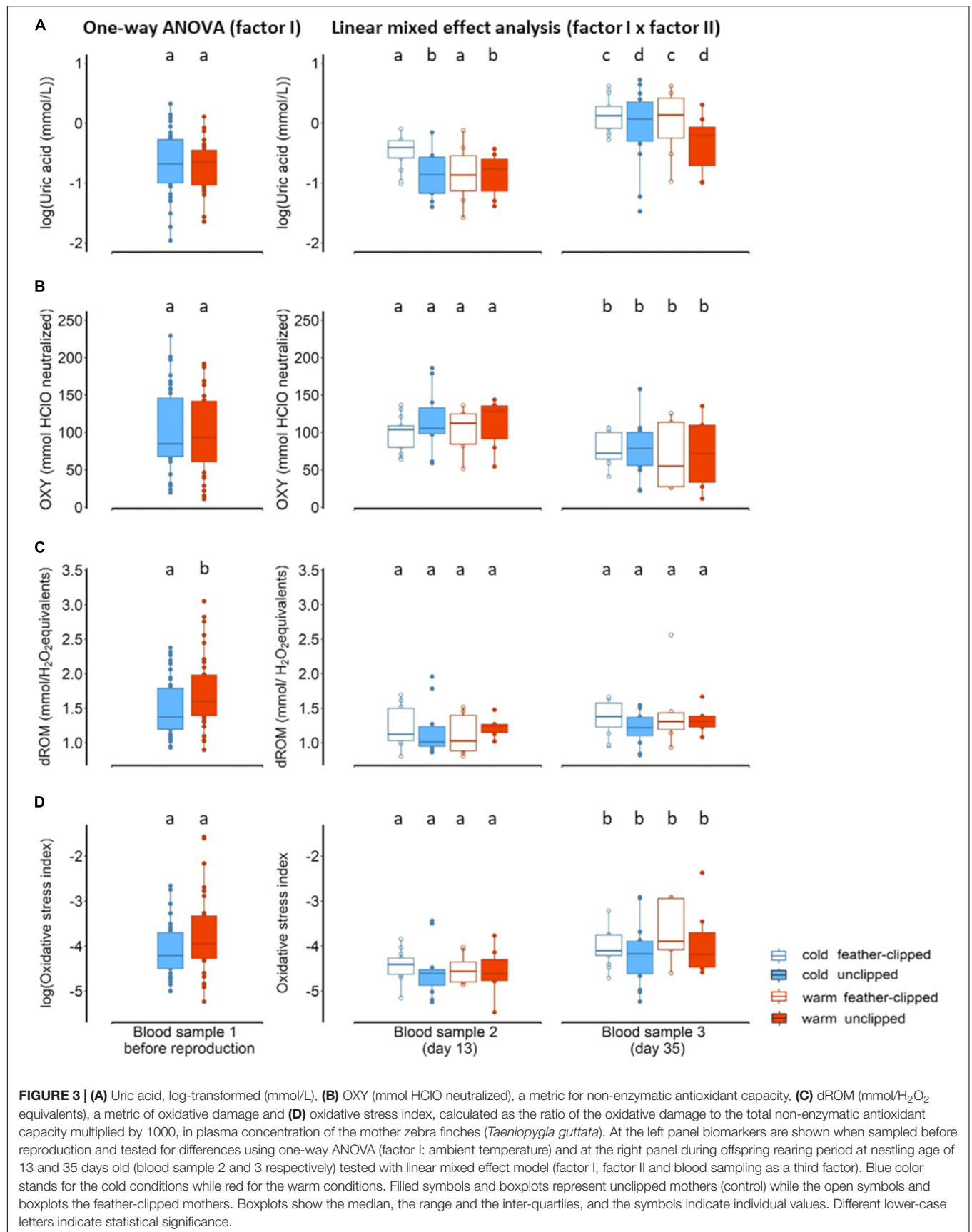


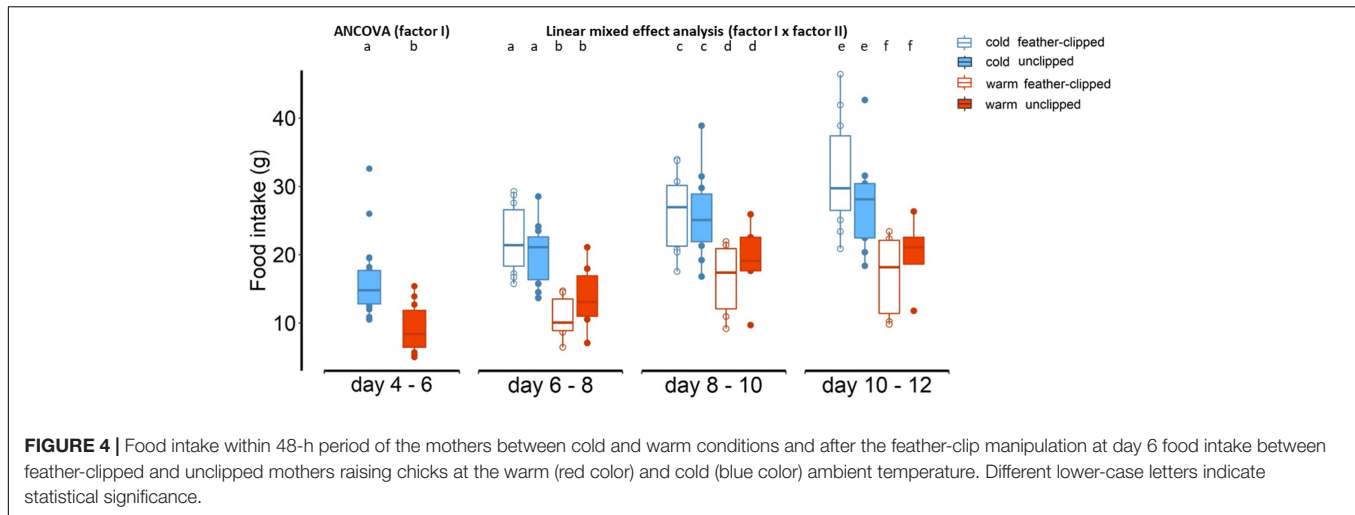
reproduction in comparison to the peak of offspring rearing period (LSM $\pm$ SE: 106.6 ± 5.4) (**Figure 3B**). However, neither ambient temperature ($p > 0.05$) nor feather-clip manipulation ($p > 0.05$) explained variation in antioxidant capacity. The effect of ambient temperature on oxidative damage was statistically unclear ($F_{1,36.5} = 0.17$, $p = 0.67$) and similarly the effect of feather-clip manipulation ($F_{1,35.9} = 1.1$, $p = 0.29$). There was weak evidence that oxidative damage differed between the two sampling points ($F_{1,40.1} = 3.1$, $p = 0.08$); oxidative damage was slightly higher at the end of offspring provisioning period (LSM $\pm$ SE: 1.25 ± 0.05) on day 35 than during the peak of offspring provisioning period (LSM $\pm$ SE: 1.13 ± 0.05) on day 13 (**Figure 3C**). Oxidative stress index (after log transformation) did not differ between ambient temperature groups ($F_{1,37.39} = 0.87$, $p = 0.35$) and feather-clip manipulation groups ($F_{1,36.58} = 1.34$, $p = 0.25$) but differed between the sampling points ($F_{1,36.9} = 21.91$, $p < 0.001$); oxidative stress index was higher toward the end of offspring-rearing period (LSM $\pm$ SE: -4.0 ± 0.09) compared to the

peak of offspring-rearing period (LSM $\pm$ SE: -4.55 ± 0.09 ; **Figure 3D**).

Maternal Trait

We found statically clear differences in food intake (within 48-h period) between the two ambient temperature groups ($F_{1,32} = 15.41$, $p < 0.001$; **Figure 4**); food intake in mothers raising hatchlings 4 to 6 days old at the warm temperature was lower (9.4 ± 1.4 g; LSM $\pm$ SE) than food intake in mothers raising hatchlings from 4 to 6 days old at the cold temperature (16.1 ± 0.9 ; LSM $\pm$ SE). After the implementation of the feather-clip manipulation, there were statistically clear differences in food intake between the two ambient temperatures ($F_{1,31.5} = 25.59$, $p < 0.001$) and the three sample points ($F_{1,84.5} = 46.59$, $p < 0.001$). However, there was no evidence that feather-clip manipulation had an effect in food intake from the mothers ($F_{1,31.5} = 0.01$, $p = 0.91$). There was no evidence for a three-way interaction between factor I, factor II and sample point ($p = 0.44$). Brood size explained most of the variation in food





intake ($F_{1,34} = 20.5$, $p < 0.001$). Food intake of the females increased along the development of the chicks, and it was lower for the females breeding in the warm conditions compared to the higher food intake of the females breeding in the cold conditions (Figure 4).

Offspring Traits

There is no evidence that body mass of the hatchlings differed between the ambient temperature conditions ($F_{1,47.2} = 0.27$, $p = 0.6$) from when eggs hatched (day 0) until the day of the feather-clip manipulation (day 6). The interaction between ambient temperature and nestling age was statistically unclear ($F_{1,271.3} = 0.74$, $p = 0.47$). There was strong evidence that body mass of the nestlings differed with the nestling age ($F_{1,271.3} = 491.54$, $p < 0.001$) and there was moderate evidence that the brood size had an effect on body mass ($F_{1,55.37} = 4.29$, $p = 0.04$).

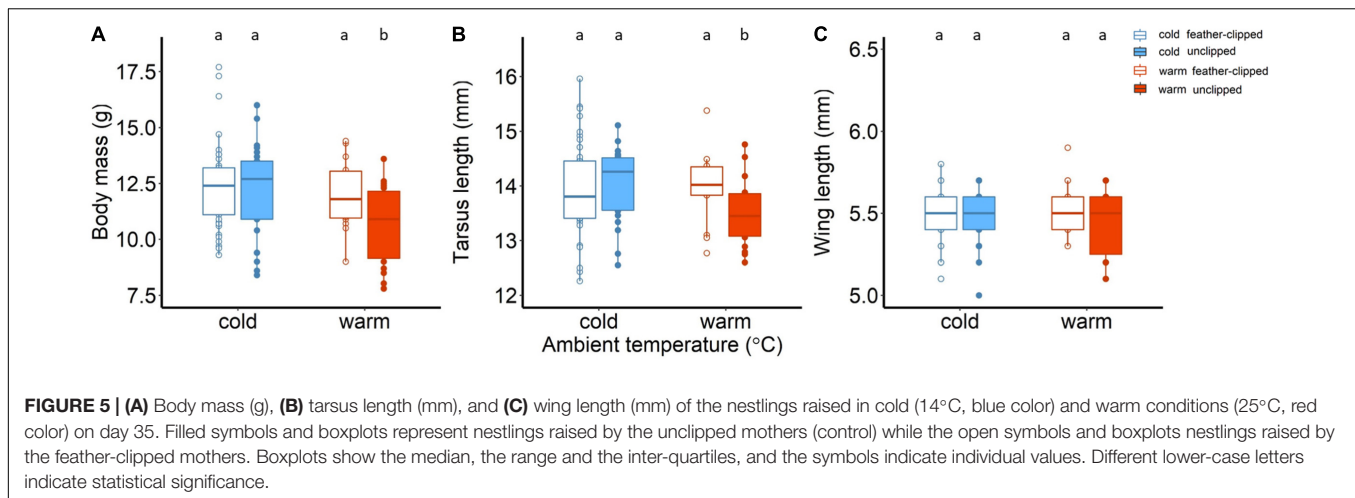
After the feather clip manipulation (day 6), we found moderate evidence for an interaction between ambient temperature (factor I), feather-clip manipulation (factor II) and the nestling age (factor III); factor I $\times$ factor II $\times$ factor III ($F_{4,465} = 3.64$, $p = 0.01$, see **Supplementary Figure 5B**). At the age of 35 days old, nestlings raised from unclipped females at warm ambient temperature had lower body mass compared to feather clipped females at the warm temperature compare to all three other groups, while no differences were detected during the nestling development (Figure 5A and **Supplementary Table 4**). Chick mass depended also on the brood size ($F_{1,39.8} = 5.8$, $p = 0.02$; **Supplementary Table 5**).

For the tarsus length of the chicks, we detected statistically clear interaction between ambient temperature and feather-clip manipulation (factor I $\times$ factor II, $F_{1,115.5} = 9.07$, $p = 0.003$). Chicks raised from the unclipped females at warm conditions had smaller tarsus length (mm) in comparison to the other three groups; warm feather-clipped, cold unclipped and cold feather-clipped (Figure 5B). Tarsus length was positively related to nestling age (tarsus length increased with increasing age from 16 to 35 days old; $F_{1,102.97} = 88.96$, $p < 0.001$), while brood

size on day 35 did not explain any variation of tarsus length ($F_{1,117.06} = 9.07$, $p = 0.76$). On the other hand, wing length was not affected by either of the two manipulations (factor I and factor II) but significantly related to nestling age; wing length was higher with increasing age (from 16 to 35 days old; $F_{1,114.59} = 2233.67$, $p < 0.001$).

DISCUSSION

First signs of reproductive limitations in line with the heat dissipation limit hypothesis (HDL, Speakman and Król, 2010b) appeared on the level of reproductive performance of both parents, in that breeding pairs at warm temperature were less successful in producing a clutch compared to the breeding pairs at the cold (Figure 1A). Egg production in terms of egg number and mass was significantly lower under warm compared to cold ambient temperature (Figures 1B,C), which is consistent with previous findings revealing decreased egg size and mass (Williams and Cooch, 1996; Schaper and Visser, 2013) with increasing ambient temperatures (but see Griffith et al., 2020). In addition, breeding zebra finches showed decreased egg mass and clutch size when exposed to a high foraging cost feeder similar to the one we implemented (Yap et al., 2021). Here, we suggest that reduced clutch size and egg mass in the warm conditions resulted from a combination of both warm temperature and elevated foraging costs. Physical exercise increases heat production and thus combined with warm ambient temperature during reproduction resulted in lower reproductive output suggesting that the capacity of heat dissipation may indeed be important. By feather-clipping females around the brood patch and the pectoral muscles, we enhanced heat dissipation capacity up to 0.03 W (9.5%) compared to the unclipped females (for further details see **Supplementary Material**). Results on maternal reproductive performance clearly support HDL hypothesis with unclipped females revealing lower body mass maintenance compared to the females with increased heat loss rate. Warm ambient temperature combined with enhanced physical activity appeared to negatively affect oxidative



damage in females prior to reproduction, whereas oxidative stress biomarkers were unaffected by both treatments, temperature, and feather-clip manipulation, during and toward the end of the reproductive activities. Even though food intake did not differ significantly between the clipped and unclipped groups, the variation might be masked by the food intake obtained by the mother and lack of direct measurements of food passed on to the nestlings. Offspring development was affected indirectly by our maternal manipulations of heat dissipation capacity, with smaller size and lighter mass of nestlings raised by the unclipped – and thus constrained in heat dissipation – mothers compared to the clipped mothers, corroborate evidence on HDL hypothesis. Our two-factorial experimental design indicates that warm ambient temperature limits energy expenditure during highly intensive activities like reproduction and that experimentally releasing the heat burden through feather clipping does relax the thermal limitations. Heat dissipation capacity appears as limitation of sustained performance.

Heat Dissipation Limitations in Breeding Females

All mothers gradually decreased their body mass during nestling rearing period (**Supplementary Figure 5A**), but un-manipulated mothers in the warm revealed lower body mass when nestlings reached 16 days old compared to the other three groups of mothers eased of heat constraints (**Figure 2A**). This suggests that unclipped mothers in the warm were limited by the capacity to heat dissipate. The differences in body mass cannot be explained from the food intake, since food intake, even though dependent on ambient temperature, was independent of the feather-clip manipulation (**Figure 4**). Body mass loss during reproduction has been previously observed in birds and it has been formulated as a consequence of either a consumption of the energy reserves (“cost of reproduction” hypothesis) or an adaptation to lower the flight costs of adults during nestling rearing (“mass-adjustment” hypothesis) (Hillström, 1995). In this study, birds had unlimited access to food during a 13 h-period of daylight and thus the hypothesis that birds were not able to

replenish their energy stores may not be supported by the “cost of reproduction” hypothesis. Maternal feather-clip manipulation in blue tits (*Cyanistes caeruleus*) enhanced heat loss by 0.09 W and revealed lower body mass loss in feather-clipped females compared to the unclipped ones when nestlings were at 14 days old (Nord and Nilsson, 2019). This is in line to the results of our present study, even though the difference in heat loss is smaller (0.03 W). Higher energy expenditure induced by an experimentally enlarged brood size in great tits (*Parus major*) was negatively related to body mass (Nadav, 1984), and Nord and Nilsson (2019) suggested that unclipped females may have lost more body mass due to the higher energetic costs of panting to dissipate heat compared to the feather-clipped ones. Panting is a known mechanism in passerines for heat dissipation (McKechnie and Wolf, 2019) and more likely to take place during high ambient temperatures (Oswald et al., 2021), and during singing increasing heat loss (Pessato et al., 2020). Panting requires a high amount of water expenditure causing changes in blood chemistry (Calder and Schmidt-Nielsen, 1966; Schmidt-Nielsen, 1972), and perhaps unclipped mothers in the warm in our study may have used more energy to dissipate heat via panting (or via singing) compared to the clipped mothers in the warm (and both experimental groups in the cold) explaining the body mass difference. We did observe that birds in the climatic chamber with warm temperature were more likely to sing than birds in the cold climatic chamber (pers. observations), but such observations require further experimental testing. Also, body temperature rises during exercise (Ward et al., 1999; Guillemette et al., 2016) and this rise becomes more pronounced with increasing ambient temperatures (Tucker and Noakes, 2009; Tapper et al., 2020a). When body temperature reaches lethal levels as shown in tree-swallows during offspring provisioning period (i.e., 15% of all observations exceeded 43°C; Tapper et al., 2020a), organisms have evolved different mechanisms to avoid any irreversible physiological damage (i.e., pacing strategy in human athletes; see review in Tucker and Noakes, 2009). Eider ducks (*Somateria mollissima*) also stop their flights when body temperature rises (Guillemette et al., 2016). Lowering the pace may explain high body mass loss in unclipped birds at the warm temperature.

Unclipped females breeding in warm conditions, hence with heat dissipation limitations, may have lost body mass as a strategy to lower metabolic heat production. For instance in humans, runners with lower body mass revealed an advantage of decreased heat production and thus were able to run faster than heavy runners under the hot ambient temperature of 35°C (Marino et al., 2000). In summary, the body mass changes of mothers in response to two manipulations provide strong experimental support that the capacity to dissipate heat may indeed represent a limit during sustained parental workload.

Enhancing the Ability to Heat Dissipate and Oxidative Stress

Since metabolic rate is affected by ambient temperature (Scholander et al., 1950), production of reactive oxygen species (ROS) is likely to be also affected by environmental temperature (Selman et al., 2000; Blagojević, 2007; Monaghan et al., 2009). In this study, after 3 weeks of acclimation to the climatic conditions, zebra finches were sampled just before reproduction and revealed higher levels of oxidative damage at the warm ambient temperature than the cold temperature (Figure 3C). Acute exposure in high ambient temperatures and/or simultaneous physical activity may induce oxidative stress (Lin et al., 2006; Mestre-Alfaro et al., 2012). Exercise produces heat and in combination with warm ambient temperature likely caused a surplus of body heat; this may explain the higher levels of oxidative damage compared to the cold we found before the reproductive event. However, oxidative damage and non-enzymatic antioxidant capacity during chick-rearing period appeared unaffected by both manipulations, such as ambient temperature and feather clipping. Mongolian gerbils (*Meriones ungulicatus*) exposed to 10°C, 21°C, and 30°C during reproduction also did not reveal differences in various markers of oxidative stress (Yang et al., 2013), similar outcome to this study. High levels of oxidative damage were detected in zebra finches when exposed in high temperatures (38°C and 42°C) during their adulthood, but this effect did not appear in birds that experienced heat-exposure in their early life (Costantini et al., 2012). Previous experience or duration (acute or chronic) of the exposure to the temperature may thus explain the fact that we detected differences in oxidative damage at the early stage of the experiment but not later during the reproductive events. The oxidative damage that birds experienced at the beginning, through increased physical activity at warm ambient temperatures, may have driven changes in the line of the antioxidant defense mechanisms, such as the non-enzymatic antioxidant capacity and uric acid (Ji, 1999; Stier et al., 2019).

Non-enzymatic antioxidant capacity of females in this study was lower toward the end of nestling-rearing period compared to the peak regardless of ambient temperature and feather-clipping (Figure 3B), confirming that the antioxidant capacity can be up-regulated for the reproductive activities (Costantini et al., 2014a). During intensive physical activities such as reproduction, organisms use antioxidants for maintaining oxidative balance. *Ad libitum* access to food in this study gave the opportunity of the mothers to upregulate their antioxidant defenses (Speakman

and Garratt, 2014), thus avoiding the risk of further oxidative stress. An opposite pattern in uric acid was detected with higher uric acid toward the end of reproduction. In birds, uric acid has been suggested to act as endogenously produced antioxidant when oxidative stress is encountered (Tsahar et al., 2006; Alan and McWilliams, 2013). For example, uric acid was found to be positively related with allantoin (its' oxidative product) in white-crowned sparrows during intensive exercise (Tsahar et al., 2006). High levels of uric acid were observed in godwits prior to migratory flight, which perhaps was caused from the breakdown of proteins that originate from body tissue (Gutiérrez et al., 2019). Here, when the up-regulated antioxidant capacity was reduced, then the uric acid took over toward the end of the reproduction and most of the variation was explained mainly by the feather-clip treatment regardless of the ambient temperature (Figure 3A). Feather-clipped females due to higher energetic investment to raise large and heavier offspring (higher reproductive output) entailed catabolism of proteins possibly due to lower investment for self-maintenance, and uric acid activated to diminish any further physiological costs. In conclusion, both oxidative stress biomarkers, non-enzymatic antioxidant capacity and uric acid, suggest that mothers during offspring-provisioning period did encounter oxidative stress and thus drove changes for the upregulation of antioxidant capacity and uric acid to protect for further costs. However, we should be cautious when interpreting the data of oxidative stress since this study is based on a limited number of assays only in plasma, without measuring enzymatic antioxidants or ROS production and is known that different assays or tissues may bring different results (Speakman and Garratt, 2014). Feather-clip manipulation influenced uric acid, suggesting that females eased from thermal limitations may increase energetic investment toward current reproduction, but this may also come with a cost of encountering oxidative stress, perhaps with lower future reproduction.

Nestling Development Constraints

Maternal treatment (i.e., feather-clipping) not only affected their own body mass over the course of the reproductive event and possibly oxidative stress but also nestling development and the interactive effect was only detected at the nestling age of 35 days old (Figure 5B and Supplementary Figure 5B). At the nestling age of 35 days zebra finches reach almost a fully developed juvenile size and move toward independence from their parents (Zann, 1996). Nestling zebra finches produced in 30°C ambient temperature were smaller in body mass and tarsus length than those in 18°C; however, these differences were only visible at the age of 28 days, but not at the age of 12 days (Andrew et al., 2017). This outcome is similar with our findings, in which reduced offspring mass and size were observed at the warm temperature raised by unclipped females on day 35, when nestlings reached nearly fully developed juvenile size, and no differences were observed in the early stages of development. Parental care and offspring development are closely coupled (Ricklefs, 1984; Zann, 1996) and it is unclear if the effect during the last stage of development is due to cumulative effects over the development of the offspring or due to the difficulty to quantify the effects of maternal care at the early stages of nestling

development. For instance, the early stage of development is characterized by intensive nestling signals, such as begging calls, that may modify and influence parental care and the level of food provisioning (Leonard and Horn, 2001). Begging calls take part in young zebra finches up to 14–16 days old and after fledgling young ones are less active and beg just before feeding bout (Zann, 1996). Even though mothers were constrained by the heat dissipation capacity, they were rather modulating maternal care due to nestling signals, hence masking any effects at the early stage. Perhaps, any effects of thermal constraints during sustained parental workload are more likely to shape offspring development at the later stage of development.

Any conditions experienced during a specific biological stage very often impact the performance of the individuals at the subsequent biological stage (Blomberg et al., 2014). When zebra finches bred in two ambient temperatures of 18°C and 30°C, nestlings raised in the hot temperature were lighter and smaller at 28 days than those raised in the cold but the effect of body mass was removed when they reached 90 days old (complete juvenile size) and only the effect in tarsus remained (Andrew et al., 2017), a partial “catch-up.” Here, nestlings raised by the unclipped mothers in the warm differed significantly in body mass and tarsus length compared to the other three groups, while wing length remained unchanged (**Figures 5B,C**). We did not follow their post-juvenile development (as in the study of Andrew et al., 2017) and we do not know whether nestlings were able to catch up with increasing their body mass. Tarsus length represents skeletal size in birds and may act as a “thermal window” which may serve for heat dissipation (Speakman and Ward, 1998). For instance, Allen’s rule suggests that small morphological traits should rather reflect an adaptive response to cold climates for heat conservation and large in warm climates for higher heat loss. Reduced body size is associated with warm climates, while increased with cold ones following Bergmann’s rule (Ashton, 2002; Cunningham et al., 2013; Kruuk et al., 2015; Andrew et al., 2017; Oswald et al., 2021). However, here we found that nestlings raised by the feather-clipped females in the warm were larger than those raised by unclipped, suggesting that the mechanistic link between the climate experienced during development and the body size is likely driven by the physiological constraints of parental care and food provisioning. Wing length represents both skeletal size and feather growth, which depends on other factors like for example nutritional supply (Senar and Pascual, 1997; Oswald and Arnold, 2012; Andrew et al., 2017). Feathers require necessary nutrients for growth (Hill and Montgomerie, 1994) and nestlings in this study even though we observed differences in body mass and tarsus length, wing length was unaffected that may be explained by the similar amount of nutrients obtained through the seeds. In addition, tarsus size of the nestlings at this stage (35 days old) was more developed close to adult size (**Figures 2B, 5B**) while wings were not fully developed (see differences in female and offspring wing length; **Figures 2C, 5C**). Perhaps any effects on wing length could be detected later when wings are fully developed close to adult size. Low mass and small structural size in nestlings implies lower survival probability (Magrath, 1991; Schwagmeyer and Mock, 2008; Ronget et al., 2018) and Tapper et al. (2020b) show

evidence that nestlings with lower mass and size raised from unclipped mothers were less successful to fledge than those raised from the feather-clipped mothers. The observed effects on nestling size and mass provide strong evidence that HDL plays a pivotal role in reproductive success and thus fitness of the organisms.

We predicted that feather clipping compared to unclipped treatment would remove the thermal limitations on reproductive performance at both ambient temperatures, but we can confirm this prediction only for the high and not for the low ambient temperature. Clearly, the difference between clipped and unclipped birds for the high ambient temperature is in agreement with HDL hypothesis, but the effect of clipping at the low ambient temperature may rather hint to opposite thermal constraints. Cold ambient temperature in combination with the feather-clip manipulation may have enhanced heat dissipation so much that birds now face an additional energetic burden for thermoregulation, namely to remain normothermic and not to cool out too much (Sadowska et al., 2019). Possibly, the activity levels modulate the impact of the feather-clipping treatment and females in the cold must allocate energy to remain warm when they are not active. In high ambient temperature clipping would improve the thermoregulation of the active birds, reducing their costs of thermoregulation. The effects of clipping on the energy balance of active birds in cold temperature and of inactive birds in high temperature should be intermediate. While we observe food intake difference only between warm and cold treatment and not between the feather manipulations, we do observe body mass effects on females and offspring and structural size effects on offspring only in the difference between clipped and unclipped females at the warm ambient temperature and not in the cold. Non-enzymatic antioxidant capacity decreased for all females during nestling-rearing period (**Figure 3B**), uric acid increased, but this was more pronounced for the feather-clipped females, both in warm and cold temperature, than the control (**Figure 3A**). These results may suggest the upregulation of the antioxidant defense system could neutralize the adverse effects of oxidative stress (Alan and McWilliams, 2013; Gutiérrez et al., 2019) but the mechanism may differ between the ambient temperatures. The association between reproduction and oxidative stress is rather complex and may also act as a constrain. Experimentally induced oxidative stress in canaries (*Serinus canaria*) delayed the hatching date and affected the clutch size (Costantini et al., 2016), suggesting that indeed oxidative stress may act as a constrain during reproduction. On the other hand, even if females were able to increase energy toward reproductive output avoiding oxidative stress, it may not necessarily be an advantage of the nestlings to gain more mass or develop larger beyond the observed levels (Blanckenhorn, 2000). Thus, it would be interesting to experimentally enhance energetic demands through an enlarged brood size and investigate whether breeding animals released of thermal constraints may raise energy expenditure. To conclude, this study corroborates HDL hypothesis, but it also indicates that alternative limitations are likely occurring during reproduction and these may even entail a differential view of opposing thermal limitations between active and non-active phases.

CONCLUSION

Our study under controlled laboratory conditions provides now comprehensive support for the HDL hypothesis and adds up to the current evidence accumulated for free-living birds that also support HDL hypothesis (Nilsson and Nord, 2018; Nord and Nilsson, 2019; Tapper et al., 2020a,b). We consider the simulation of high foraging costs of free-living birds despite the typical *ad libitum* access to food in the laboratory as important requirement for meaningful testing of HDL hypothesis in captive species. Under the scenario of contemporary climate change, which is characterized by continuously rising ambient temperatures and extreme weather events such as heat waves (Ummenhofer and Meehl, 2017), thermal limitations can be expected to impose even bigger and strenuous challenges for organisms in general, and during reproduction in particular. Higher-than-normal temperatures may induce oxidative stress which also seems to be the driver for changes in the line of antioxidant defense, but further research is required to understand potential trade-offs between management of oxidative stress under thermal limitations and body condition. Oxidative stress avoidance during reproduction might be the key to understand the allocation of resources to either current or future reproduction. Any challenges due to high temperatures during reproductive events may suppress both current and future reproduction (see also Andreasson et al., 2020), and consequently setting limits to species distribution. Our research under controlled conditions provides clear understanding on thermal limitations to influence reproductive output in terms of quantity and quality. Such detailed understanding of what limits species distribution and fitness may enable us to tailor conservation approaches that are particularly needed under the current global change scenario.

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/s.

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ETHICS STATEMENT

The animal study was reviewed and approved (Decision no 143/2017 and 241/2018).

AUTHOR CONTRIBUTIONS

EZ, ES, and UB conceived the experiment. EZ performed the experiment and carried out all measurements with the help of PM-V. EZ analyzed the data and wrote the first draft. ES analyzed respiratory measurements presented at the **Supplementary Material**. All authors contributed significantly to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.866182/full#supplementary-material>

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Collapse of Breeding Success in Desert-Dwelling Hornbills Evident Within a Single Decade

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Rapid anthropogenic climate change potentially severely reduces avian breeding success. While the consequences of high temperatures and drought are reasonably well-studied within single breeding seasons, their impacts over decadal time scales are less clear. We assessed the effects of air temperature (T_{air}) and drought on the breeding output of southern yellow-billed hornbills (*Tockus leucomelas*; hornbills) in the Kalahari Desert over a decade (2008–2019). We aimed to document trends in breeding performance in an arid-zone bird during a time of rapid global warming and identify potential drivers of variation in breeding performance. The breeding output of our study population collapsed during the monitoring period. Comparing the first three seasons (2008–2011) of monitoring to the last three seasons (2016–2019), the mean percentage of nest boxes that were occupied declined from 52% to 12%, nest success from 58% to 17%, and mean fledglings produced per breeding attempt from 1.1 to 0.4. Breeding output was negatively correlated with increasing days on which T_{max} (mean maximum daily T_{air}) exceeded the threshold T_{air} at which male hornbills show a 50% likelihood of engaging in heat dissipation behavior [i.e., panting (T_{thresh} ; $T_{\text{air}} = 34.5^{\circ}\text{C}$)] and the occurrence of drought within the breeding season, as well as later dates for entry into the nest cavity (i.e., nest initiation) and fewer days post-hatch, spent incarcerated in the nest by the female parent. The apparent effects of high T_{air} were present even in non-drought years; of the 115 breeding attempts that were recorded, all 18 attempts that had $\geq 72\%$ days during the attempt on which $T_{\text{max}} > T_{\text{thresh}}$ failed (equivalent to T_{max} during the attempt $\geq 35.7^{\circ}\text{C}$). This suggests that global warming was likely the primary driver of the recent, rapid breeding success collapse. Based on current warming trends, the T_{max} threshold of 35.7°C , above which no successful breeding attempts were recorded, will be exceeded during the entire hornbill breeding season by approximately 2027 at our study site. Therefore, our findings support the prediction that climate change may drive rapid declines and cause local extinctions despite the absence of direct lethal effects of extreme heat events.

Keywords: southern yellow-billed hornbill, high temperatures, drought, breeding, climate change, arid-zone bird

INTRODUCTION

Global heating is exacerbating the harsh conditions associated with arid environments by elevating average air temperatures (T_{air}) and increasing the frequency and intensity of heatwaves (Pachauri et al., 2014; Mbokodo et al., 2020) and drought (van Wilgen et al., 2016). Possible consequences for animals inhabiting arid regions include increased frequency of mass mortality events (Welbergen et al., 2008; Albright et al., 2017; McKechnie and Wolf, 2019; McKechnie et al., 2021) and disastrous reproductive failures (Bolger et al., 2005; Albright et al., 2010; Seabrook et al., 2014; McCowan and Griffith, 2021; Sharpe et al., 2021). However, heatwaves, especially in association with droughts, may also have insidious sublethal effects (Gardner et al., 2016; Conradie et al., 2019; Bourne et al., 2020b, 2021; Cunningham et al., 2021; Moagi et al., 2021), including loss of body condition (du Plessis et al., 2012; Sharpe et al., 2019), reduced egg or clutch sizes in birds (Albright et al., 2010; Keynan and Yosef, 2010), reduced provisioning rates and compromised offspring quality (van de Ven et al., 2020a; Bourne et al., 2021; Oswald et al., 2021), and the foregoing of breeding altogether (Moreno and Møller, 2011; McCreedy and van Riper, 2015; Carstens et al., 2019).

The small size and high mass-specific metabolism of most birds, combined with a diurnal activity period and limited use of underground refugia, make this taxon among the most vulnerable to mismatches between energy and water balance over short time scales (Wolf, 2000; Albright et al., 2017; Conradie et al., 2019; Cooper et al., 2020; Kemp et al., 2020). Arid-zone birds are particularly vulnerable given that they are commonly constrained to breed in response to rainfall, which often occurs during the hottest time of the year (Bolger et al., 2005; Flesch, 2014; McCreedy and van Riper, 2015; Mares et al., 2017). Given that birds face the dual threats of dehydration and hyperthermia daily during this period, elucidating the limits of their capacity to cope with the increased resource (food, water, and time) demands associated with breeding is critical for understanding variation in reproductive success under current and future climates (Andreasson et al., 2020; Cunningham et al., 2021). There is a growing body of literature on the impacts of high temperatures and drought on nest success of arid-zone birds at the scale of nesting attempts and seasons (e.g., Catry et al., 2011, 2015; Salaberria et al., 2014; Cruz-McDonnell and Wolf, 2016; Bourne et al., 2020a,b; D'Amelio et al., 2021), but fewer studies investigate these impacts on population-level breeding outputs over longer timescales in relation to ongoing climate change trends (however, see e.g., Frederiksen et al., 2013; Hatch, 2013; Amélineau et al., 2019; Bourne et al., 2020a; Ridley et al., 2021).

The southern yellow-billed hornbill (*Tockus leucomelas*; hereafter hornbill) initiates breeding in response to rainfall in the arid western parts of its southern African range (Kemp and Woodcock, 1995; van de Ven et al., 2020a). Like most hornbills, these hornbills employ an unusual breeding strategy whereby the female seals herself into the nest cavity (Kemp and Woodcock, 1995). Nestling hornbills within the sealed nest cavity are therefore largely safe from predators, meaning that breeding success is primarily dependent on other factors

(Moreau and Moreau, 1941). In our study population, which has been monitored since 2008, the mean maximum daily T_{air} (T_{max}) during the nesting period negatively correlates with fledging probability and fledgling condition (van de Ven et al., 2020a). The reduction in breeding success during hot periods is likely caused by a combination of high nest temperatures (T_{nest}) and reduced provisioning rates by males, due to trade-offs between thermoregulation and parental effort (van de Ven et al., 2019, 2020a). Considering (a) the mechanistic links between weather conditions and breeding are well-studied in hornbills (van de Ven et al., 2019, 2020a; Van Jaarsveld et al., 2021), (b) *a priori*, the unusual breeding system of hornbills and the fact they are constrained to breed during the hottest time of year in an arid zone indicates this taxon could be especially vulnerable to global warming, and (c) a 10-year monitoring dataset exists for our study population, which resides near the hot, arid southwestern edge of the species' distribution, we suggest that this population of hornbills provides an excellent opportunity to test the prediction that lethal and sublethal fitness costs of high T_{max} will lead to increasingly poor reproductive output over time, risking breeding collapse and population extirpation due to climate change (Conradie et al., 2019).

In this study, we examined climate trends (1960–2020) for the study region and the breeding success of hornbills at both broad (long-term trends 2008–2018) and fine (individual breeding attempts) scales. Our specific aims were to (1) quantify long-term climate trends in the study region, (2) quantify changes in the breeding success of this population of hornbills over the last decade, and (3) model the relationships between several extrinsic (metrics of rainfall and temperature) and intrinsic (female entry into the nest cavity date and days post-hatch the female spent incarcerated in the nest) variables on the breeding success of hornbills. Our overall aim was to describe the response of an arid-zone bird to a decade-long period of rapid warming, identify potential drivers of variability in breeding performance, and empirically demonstrate likely mechanistic links between climate change and collapse of breeding output.

MATERIALS AND METHODS

Study Species

The southern yellow-billed hornbill is listed as the least concern by the International Union for Conservation of Nature (IUCN) but are thought to be declining (BirdLife International, 2021). The hornbill's distribution includes most of southern Africa, with a large portion falling within the Kalahari Desert. They are socially monogamous and live up to 23 years in captivity (Strehlow, 2001), although the oldest known marked bird at our study site was 8 years old. Hornbills are cavity nesters; once sealed into the nest, the females moult all their flight feathers simultaneously. At our study site, pairs usually attempt a single brood per season (though they may be multibrooded in more mesic areas, Stanback et al., 2021), and the mean duration from the first egg lay to the female completing sufficient feather regrowth and breaking out of the nest is 53 ± 6.3 SD days ($n = 56$, range: 36–73) in nests that successfully produce at

least one fledgling (van de Ven et al., 2020a). After the female breaks out of the sealed cavity, she aids in the subsequent provisioning of nestlings.

Research on hornbills has been ongoing at our study site (Kuruman River Reserve in the southern Kalahari Desert, Northern Cape province, South Africa; 26°58' S, 21°49' E; hereafter, KRR) since 2008, and breeding data for ten breeding seasons (2008–2019, excluding the summer of 2011–2012 when no data were collected) were used for this study. Hornbills at KRR typically breed between October and the end of March (van de Ven et al., 2020a). Monitoring allowed us to estimate the number of pairs at the site and track if and where they bred. During the decade monitoring period, some pairs were recorded breeding in natural cavities. No breeding data were recorded from these attempts. Breeding primarily occurred in nest boxes, and the breeding data analyzed in this study were exclusively from nest boxes. Wooden nest boxes were available at KRR throughout the study period (43 boxes during 2008–2011, 44 boxes during 2012–2015, 38 boxes during 2015–2017, 33 boxes during 2017–2018, and 36 boxes during 2018–2019). In seasons with low nest box occupancy, pairs were observed to still be present at the site but to skip breeding. We defined the hornbill breeding season as 19 October (the earliest recorded date a female entered a box during the decade of the study) to 24 March (the latest recorded fledge/failure date).

Study Site and Weather Data Collection

The KRR is an arid savanna dominated by grasses interspersed with large trees (mainly camelthorn, *Vachellia erioloba*; gray camelthorn, *Vachellia haematoxylon*; shepherds' tree, *Boscia albitrunca*; and buffalo thorn, *Ziziphus mucronata*). Rainfall occurs primarily in the Austral spring and summer between October and April [mean annual rainfall: $213.9 \pm \text{SD } 102.0$ mm; 1993–2020, Van Zylsrus (VZ) weather station, South African Weather Service (SAWS)]. The spring and summer are hot; T_{max} [mean maximum daily air temperature (T_{air})] between October and March is $34.9 \pm \text{SD } 1.19^\circ\text{C}$ (1991–2020, VZ), which is above the threshold T_{air} at which male hornbills showed a 50% likelihood of engaging in heat dissipation behavior, i.e., panting [T_{thresh} ; $T_{\text{air}} = 34.5^\circ\text{C}$] (van de Ven et al., 2019).

In 2011, the study site was equipped with a weather station [Hot Birds Research Project (HBRP) Weather Station; Vantage Pro2, Davis Instruments, Hayward, CA, United States] that was set to record T_{air} ($^\circ\text{C}$), wind speed ($\text{m}\cdot\text{s}^{-1}$), relative humidity (%), and solar radiation ($\text{W}\cdot\text{m}^{-2}$) at 5-min intervals. To create a continuous weather dataset appropriate to the study site spanning the entire hornbill monitoring period (2008–2019), we compared weather data from the HBRP station collected between 2011 and 2020 to those from SAWS stations at VZ, Northern Cape (~30 km from the study site, data available 1992–2020), and Twee Rivieren (TR), Northern Cape (~120 km from the study site, data available 1960–2020) to validate the use of VZ and TR weather data as proxies for the missing HBRP weather data prior to 2011.

Weather data from all three stations were highly correlated (see **Supplementary Figures 1, 2**), but the T_{max} data from VZ and TR were consistently higher than those from the HBRP

(VZ mean: $1.38^\circ\text{C} \pm 1.54$ SD higher; TR, mean: $2.13^\circ\text{C} \pm 1.79$ SD higher; **Supplementary Figure 1**). Therefore, these values were adjusted (refer to **Supplementary Figure 2**) before being used to supplement the HBRP weather data collected at the study site. Daily rainfall was not correlated between the three weather stations, but seasonal rainfall was observed as follows: $\text{HBRP} \sim \text{VZ } r^2 = 0.97$; $\text{HBRP} \sim \text{TR } r^2 = 0.86$ (**Supplementary Figure 3**), allowing us to establish the occurrence/absence of drought at the study site using VZ rainfall data. The mean breeding season rainfall recorded by the VZ weather station was 151.71 mm (average rainfall recorded during the hornbill breeding season was 19 October–24 March each year from 1993 to 2019). We defined “drought” seasons as those in which rainfall recorded at VZ was $< 80\%$ of the long-term seasonal average following Bourne et al. (2020a). Rainfall has a lagged effect on prey abundance at the study site (Doolan and Macdonald, 1997), therefore the cumulative rainfall within the 2 months prior to the start of the breeding season was included in analyses as a proxy for food availability during breeding (Ridley and Raihani, 2007). To analyze long-term weather trends in southern Kalahari, we used unadjusted weather data from all three weather stations.

Statistical Analyses

Long-Term Weather Trends

Statistical analyses were conducted in the R statistical environment, version 3.5.1 (R Core Team, 2021), using the R Studio platform (R Studio Team, 2021). For each weather station (HBRP, TR, and VZ), we used generalized linear models (GLMs) to model yearly trends in the T_{max} (TR: 1960–2020; VZ: 1990–2020; and HBRP: 2011–2020), cumulative days on which $T_{\text{max}} > T_{\text{thresh}}$ (Days T_{thresh} ; TR: 1960–2020; VZ: 1990–2020; and HBRP: 2011–2020), and cumulative rainfall (TR: 2007–2020; VZ: 1995–2020; and HBRP: 2011–2020), for each hornbill breeding season (19 October–24 March annually). A visual inspection of the T_{air} data from TR indicated a non-linear relationship. Therefore, we used segmented analysis (package *segmented*, Muggeo, 2017) on the dataset with the longest record (TR, starting 1960) to investigate whether there was a statistically significant inflection in seasonal T_{max} and cumulative Days T_{thresh} each season. We also modeled the yearly trends in the non-breeding season (25 March–18 October each year) mean minimum daily T_{air} (T_{min} ; TR: 1960–2020; VZ: 1990–2020; and HBRP: 2011–2020) and cumulative rainfall (TR: 2007–2020; VZ: 1995–2020; HBRP 2011–2020). Only breeding and non-breeding seasons with data covering $> 90\%$ of the days were used in analyses (for excluded seasons see **Supplementary Table 1**). All trends were plotted with the linear model predictions (package *ggplot2*, Wickham, 2016).

Seasonal Weather Conditions and Breeding Trends

We tested for temporal autocorrelation in all response variables across seasons by assessing the degree of correlation between the model residuals associated with increasing lags within each model. As no temporal autocorrelation between seasons was found for any variables, and sample size in terms of number of seasons was small ($n = 10$), we used simple Pearson's product-moment correlation coefficient tests to assess the statistical

significance of the following long-term trends: (1) seasonal $T_{\max}$ and cumulative rainfall for breeding seasons 2008–2019; (2) percentage of nest boxes used, percentage of nesting attempts that succeeded in fledging at least one chick (hereafter succeeded), and mean number of fledglings produced per breeding attempt per season 2008–2019; and (3) relationships between the following weather variables: breeding season (18 August–18 October annually) cumulative $\text{Days}T_{\text{thresh}}$ (this metric used since panting is associated with heavy reductions in foraging success (van de Ven et al., 2019), making $\text{Days}T_{\text{thresh}}$ a biologically instructive metric for analyses); breeding season drought occurrence; non-breeding season $T_{\min}$; and the cumulative rainfall within the 2 months prior to the start of the breeding season, and (a) percentage of boxes used per season (b) percentage of nesting attempts that succeeded, and (c) the mean number of fledglings produced per attempt per season. Simple linear trends were then visualized using *ggplot2* (Wickham, 2016). We also performed Welch two-sample *t*-tests on each of these dependent variables as a function of drought occurrence.

Individual Breeding Attempts

A total of 115 breeding attempts were recorded in nest boxes over the study period, of which 91 succeeded. Complete or partial weather data were available for 109 breeding attempts, and female entry date was known for 105. Due to these data gaps, the final sample sizes for analyses of nest survival in relation to weather conditions were $n = 88$ for (a) female entry to fledge/fail (hereafter “entire attempt”) and (b) female entry to hatch/fail (hereafter “pre-hatch period”). Attempts that had the required combination of known hatch date ($n = 91$) and complete weather data allowed for a final sample size of $n = 88$ for analyses of (c) hatch to nestling fledge/fail (hereafter “nestling period”).

Bayesian generalized linear mixed models (BGLMer) with binomial distribution were used to model the success/failure of (a) each entire attempt, (b) the pre-hatch period, and (c) the nestling period, as a function of the female entry date (coded as the count of days since the start of the breeding season, with 19 October = day 0); the percentage $\text{Days}T_{\text{thresh}}$ during the attempt; drought occurrence; and the interaction between the percentage $\text{Days}T_{\text{thresh}}$ and drought occurrence. The number of days the female spent incarcerated in the nest after hatching was included as an additional explanatory variable for nestling period analyses. We used Bayesian linear mixed models (BLMer) to model the effect of drought occurrence on female entry date and on the number of days post-hatch spent in the nest by the female, with ANOVA (Type II Wald chi-square test) used to test for significant correlations with drought occurrence. Season identity was included in all models as a random effect.

We used variance inflation factors (VIF; package *car*, Fox and Weisberg, 2019) to test for collinearity between explanatory variables and did not fit correlated variables within the same model. We used percentage $\text{Days}T_{\text{thresh}}$ of each nesting period as our “temperature” predictor variable rather than $T_{\max}$ of each nesting period as our “temperature” predictor variable because these two variables showed notable collinearity in all models ($\text{VIF} > 10$), and models including only percentage $\text{Days}T_{\text{thresh}}$ outperformed models including only $T_{\max}$ according to Akaike information criterion (AICc) adjusted for small sizes.

We used “drought occurrence” (number of breeding attempts in drought years = 28, mean rainfall = 75.0 ± 42.8 mm, number of breeding attempts in non-drought years = 86, mean rainfall = 257.0 ± 29.1 mm) as our “precipitation” predictor variable instead of cumulative rainfall during the attempt because daily rainfall data were available for only 36 of 115 individual breeding attempts.

For each analysis, candidate model sets included all possible nested models within a biologically sensible global model, including the null model. We used a Bayesian modeling approach since it allowed us to fit a global model with the *a priori* terms of interest while avoiding singularity errors (package *blme*, Chung et al., 2013). Candidate models were compared using AICc (package *MuMIn*, Barton, 2020). We reported on the top-performing model, and where more than one top model was identified (those with an $\Delta \text{AICc} < 2$), results were reported for the model average of the top models (package *MuMIn*, Barton, 2020). The plots were based on the single model with the lowest AICc (package *jtools*, Long, 2020).

Thermal Conditions in Nest Boxes vs. Natural Cavities

All our breeding success data were necessarily collected from birds breeding in nest boxes (as opposed to natural tree cavities, which were sealed and inaccessible). However, between 2012 and 2015, we collected nest temperature (T_{nest}) data from occupied natural cavities to compare thermal conditions in these two nest types. We analyzed T_{nest} data from 27 occupied nest boxes and 9 occupied natural cavities (cumulatively 58,632 h and 9,816 h recording at 5-min intervals, respectively) collected between 2012 and 2015 using Thermochron iButtons (DS1923, Maxim, Sunnyvale, CA, United States, resolution = 0.0625°C). For nest box measurements, iButtons were mounted within the nests on the underside of the nest box lids in plastic iButton wall holders to minimize bird contact and to avoid interference by the birds. For natural cavity measurements, iButtons were placed in an iButton reader holster, which was secured with threading wire at the top of the cavity *via* the nest opening. We used Bayesian linear mixed models (BLMers) to model T_{nest} as a function of T_{air} as measured by the HBRP weather station, nest type (box vs. cavity), and the interaction between those factors. Individual nest identity was included as a random factor. ANOVA (Type II Wald chi-square test) was used to identify factors with significant correlations with T_{nest} .

RESULTS

Long-Term Weather Trends

In the TR weather data, there was a significant inflection in the summer of 1996–1997, after which $T_{\max}$ ($t_{54} = 4.58$, $p < 0.001$, ± 3.5 SE years) and the cumulative $\text{Days}T_{\text{thresh}}$ ($t_{54} = 3.9$, $p < 0.001$, ± 4.1 SE years) began to increase (Figures 1A,B). After summer 1996–1997, TR mean seasonal $T_{\max}$ increased by 1.3°C per decade and cumulative $\text{Days}T_{\text{thresh}}$ by 19.7 days per decade. We did not test for an inflection point in the VZ $T_{\max}$ data; rather, considering the strong

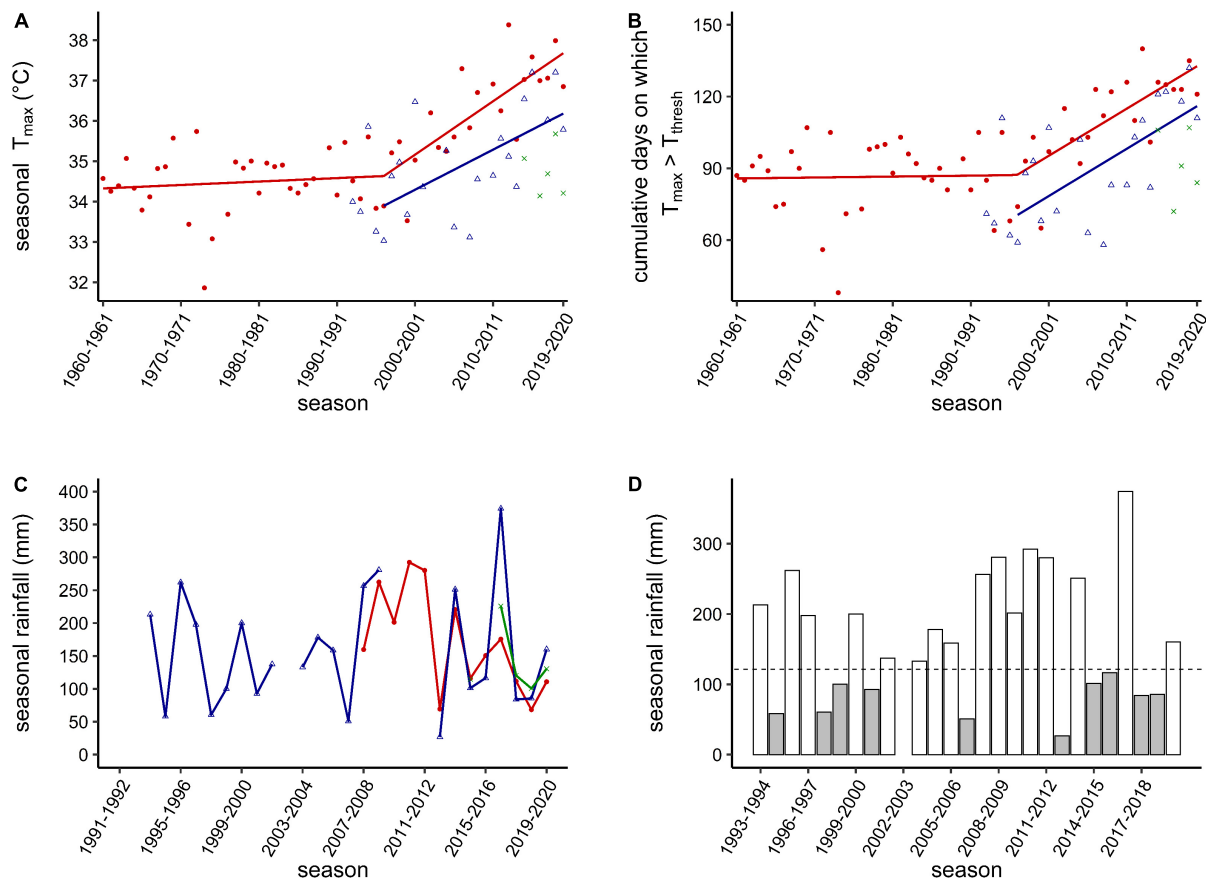


FIGURE 1 | (A) Trends in seasonal T_{max} [mean maximum daily air temperature (T_{air})], **(B)** cumulative $DaysT_{thresh}$ [days on which $T_{max} > T_{thresh}$ ($T_{air} = 34.5^{\circ}\text{C}$)], and **(C)** cumulative rainfall for each southern yellow-billed hornbill (*Tockus leucomelas*) breeding season (19 October–24 March each year) modeled from Hot Birds Research Project (HBRP; the on-site weather station, green crosses), Twee Rivieren (red circles, ~120 km from the study site), and Van Zylsrus (blue triangles, ~30 km from the study site) weather station data. A significant increase in T_{max} (± 3.5 SE years) and cumulative $DaysT_{thresh}$ (± 4.1 SE years) starting in approximately 1996–1997 was identified using segmented analysis for the TR weather station. **(D)** Bar plot for cumulative rainfall from the VZ weather station (data from TR between 2009 and 2012 included where VZ rainfall data were unavailable) with a dotted line showing the threshold for drought (gray bars) vs. non-drought (clear bars) years.

correlation between TR and VZ T_{max} data ($\rho = 0.94$, $r^2 = 0.89$; **Supplementary Figure 1**), we applied the same inflection point identified for the TR data to the VZ data. Above the same inflection point, the VZ mean seasonal T_{max} increased 1.0°C per decade and the cumulative days on which $T_{max} > T_{thresh}$ by 19.8 days per decade. Too few years of data were available for the HBRP station to model a trend in T_{max} ; however, given the strong correlation between the VZ and HBRP data ($\rho = 0.94$, $r^2 = 0.94$, **Supplementary Figure 1**), a similar warming trend of 1.0°C per decade can be expected for HBRP. No clear long-term pattern in rainfall was identified for either station; however, rainfall records only date back to 1993 (**Figures 1C,D**). No trends or inflection points were apparent in T_{min} or cumulative rainfall during the non-breeding season since 1960 and 1995 for TR, respectively (**Supplementary Figure 4**).

Long-Term Breeding Trends

The percentage of nest boxes used ($r = -0.80$, $p = 0.005$) and the percentage of nesting attempts that succeeded ($r = -0.73$,

$p = 0.017$) per season declined over the course of the monitoring period (**Table 1** and **Figure 2**). There was a marginally non-significant decline in the mean number of fledglings produced per breeding pair per season ($r = -0.63$, $p = 0.051$, **Table 1**). We found no evidence of temporal autocorrelation in the number or percentage of nest boxes used, the percentage of nesting attempts that succeeded, or the mean fledglings per attempt, modeled by season.

The percentage of boxes used ($r = -0.88$, $p < 0.001$), percentage of successful attempts ($r = -0.90$, $p < 0.001$), and mean fledglings per attempt ($r = -0.78$, $p = 0.008$) all declined significantly with increasing cumulative $DaysT_{thresh}$ and were all significantly higher in non-drought seasons compared to drought seasons ($p = 0.014$, $p \leq 0.001$, and $p = 0.005$, respectively; **Table 1** and **Figure 3**). However, neither percentage of boxes used ($r = -0.43$, $p = 0.220$; $r = 0.27$, $p = 0.451$), the percentage of successful attempts ($r = -0.59$, $p = 0.075$; $r = 0.15$, $p = 0.670$), nor the mean fledglings produced per attempt ($r = -0.56$, $p = 0.092$; $r = 0.22$, $p = 0.537$) were significantly related to

TABLE 1 | Pearson's correlation test results for the percentage of boxes used, percentage of attempts fledging at least one chick, and mean fledglings per attempt per season in the southern yellow-billed hornbills (*Tockus leucomelas*), correlated with the cumulative Days T_{thresh} {days on which T_{max} [mean maximum daily air temperature (T_{air})] $> T_{\text{thresh}}$ ($T_{\text{air}} = 34.5^{\circ}\text{C}$) within the breeding season (19 October–24 March), T_{min} (mean minimum daily T_{air}), and cumulative rainfall within the 2 months prior to the start of the breeding season (18 August–18 October each year).

Response variable	Independent variables	Pearson's corr. r	t	df	$p \leq$	Lower 95% CI	Upper 95% CI
% boxes used	Season	−0.80	−3.82	8	0.005	−0.95	−0.35
	Cumulative Days T_{thresh}	−0.88	−5.32	8	0.001	−0.97	−0.57
	Preceding 2 months' cumulative rainfall	−0.43	−1.33	8	0.220	−0.83	0.28
	Drought occurrence		−3.13	8	0.014	−54.56	−8.30
	T_{min} (non-breeding season)	0.27	0.79	8	0.451	−0.43	0.77
% success	Season	−0.73	−3.00	8	0.017	−0.93	−0.18
	Cumulative Days T_{thresh}	−0.90	−5.81	8	0.001	−0.98	−0.62
	Preceding 2 months' cumulative rainfall	−0.59	−2.05	8	0.075	−0.89	0.07
	Drought occurrence		−7.89	8	0.000	−65.82	−36.01
	T_{min} (non-breeding season)	0.15	0.44	8	0.670	−0.53	0.71
Mean fledglings per attempt	Season	−0.63	−2.29	8	0.051	−0.90	0.00
	Cumulative Days T_{thresh}	−0.78	−3.50	8	0.008	−0.94	−0.29
	Preceding 2 months' cumulative rainfall	−0.56	−1.92	8	0.092	−0.88	0.11
	Drought occurrence		−5.15	8	0.005	−1.53	−0.49
	T_{min} (non-breeding season)	0.22	0.65	8	0.537	−0.47	0.75

Welch Two Sample t-test results for the same three response variables as a function of drought occurrence over the breeding season are also shown. Rows for independent variables with a significant effect are in bold.

the cumulative 2 months' rainfall preceding the start of the breeding season or the T_{min} of the preceding non-breeding season, respectively (Table 1).

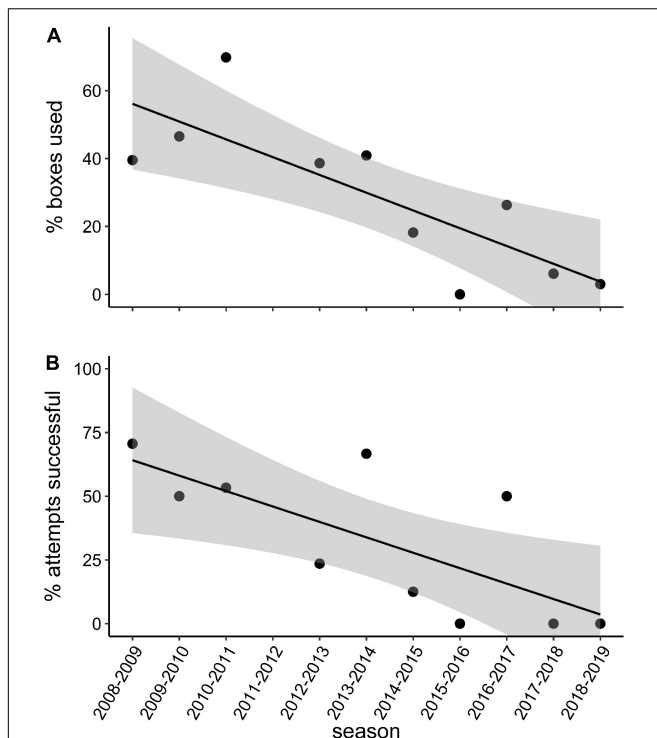


FIGURE 2 | (A) The percentage of available nest boxes occupied and **(B)** the percentage of breeding attempts that succeeded per season in our study population of the southern yellow-billed hornbills (*Tockus leucomelas*) between 2008 and 2019. Both trends are significant ($p < 0.05$); 95% confidence intervals are shown.

Individual Breeding Attempts

One best-performing model was identified for variation in breeding success (model weight: 0.96, **Supplementary Table 2**). This model indicated that the probability of success was negatively correlated with later female entry dates (estimate: -1.10 ± 0.41 ; $p = 0.007$; **Table 2** and **Figure 4**). Additionally, there was a significant interaction between the percentage Days T_{thresh} during the attempt and the drought occurrence on breeding success (estimate: -2.65 ± 1.04 ; $p = 0.011$, **Table 2**). *Post hoc* models of breeding success in drought and non-drought years revealed a significant negative correlation between the percentage Days T_{thresh} during the attempt and breeding success in non-drought seasons (estimate: -1.87 ± 0.65 ; $p = 0.004$), but not in drought seasons (estimate: -0.20 ± 0.67 ; $p = 0.771$, **Table 2** and **Figure 4**). In drought seasons, breeding success was uniformly low; only four of 23 (17.4%) attempts succeeded in fledging at least one chick, compared to 40 of 65 (61.5%) in non-drought seasons. Of the 115 breeding attempts, we recorded all 18 attempts that experienced $\geq 72\%$ of the days on which $T_{\text{max}} > T_{\text{thresh}}$ (corresponding to T_{max} during the attempt $\geq 35.7^{\circ}\text{C}$) failed (**Figure 4**). Based on current warming trends of 1°C per decade (**Figure 1**), this T_{max} threshold of 35.7°C will be exceeded throughout the entire breeding season by approximately 2027 at our study site.

One top model was identified for the probability of a breeding attempt successfully progressing from female entry to hatch (model weight: 0.66, **Supplementary Table 2**), with nests significantly less likely to progress to hatching when females entered the nest later (estimate: -1.24 ± 0.44 ; $p = 0.005$, **Table 2**). The interaction between the percentage Days T_{thresh} during the attempt and drought occurrence was significant (estimate -1.55 ± 0.77 ; $p = 0.005$, **Table 2**); the probability of a breeding attempt successfully progressing from female entry to hatch declined significantly with an increasing percentage

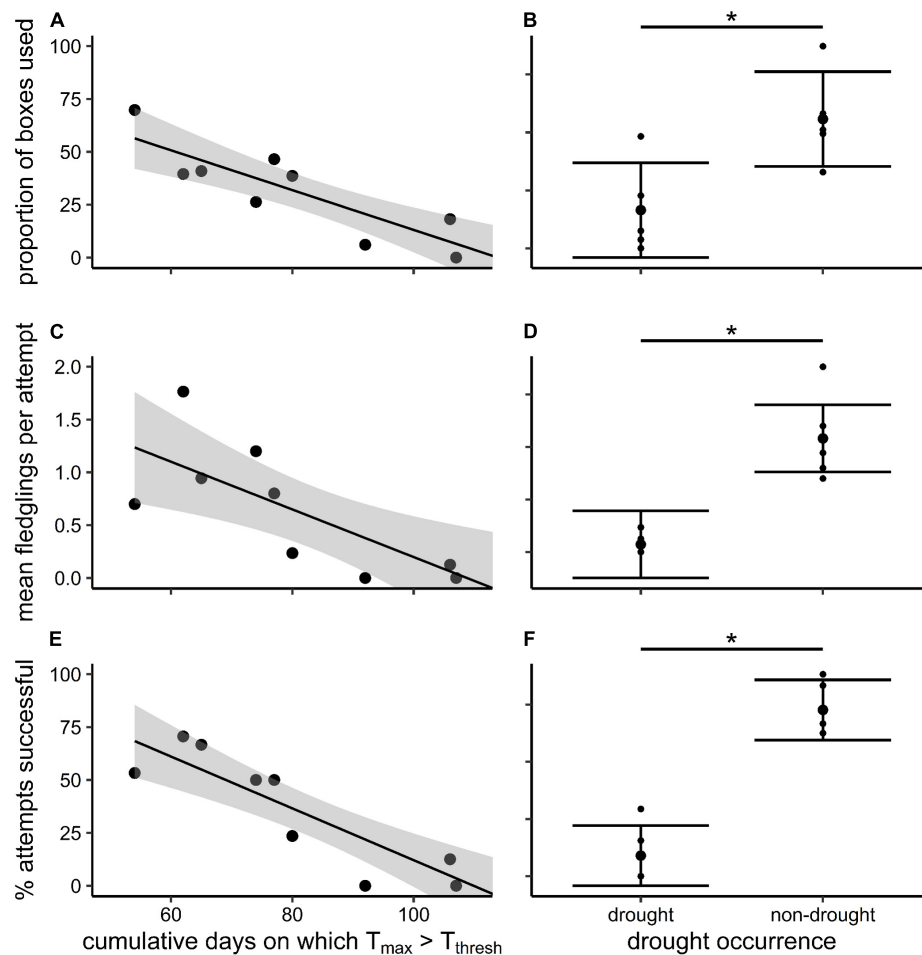


FIGURE 3 | (A,B) The percentage of boxes used, **(C,D)** the mean fledglings per attempt, and **(E,F)** the percentage of attempts that succeeded in the southern yellow-billed hornbills (*Tockus leucomelas*) as functions of the cumulative Days T_{thresh} {days on which $T_{\max}$ [mean maximum daily air temperature (T_{air})] $> T_{\text{thresh}}$ ($T_{\text{air}} = 34.5^{\circ}\text{C}$)}, and occurrence of drought across the breeding season. All trends are significant ($p < 0.05$), and 95% confidence intervals and means with upper and lower quartiles are shown.

Days T_{thresh} during the pre-hatch period within non-drought seasons (estimate: -1.22 ± 0.00 ; $p = 0.013$), but not within drought seasons (estimate: -0.91 ± 0.97 ; $p = 0.310$; **Table 2**) where successful hatches were low; 15 out of 23 (65.2%) attempts hatched in drought seasons, compared to 56 out of 65 (86.2%) in non-drought seasons.

During the nestling period, four top models for breeding attempt success were identified (model weights: 0.30, 0.20, 0.13, and 0.11 respectively; **Supplementary Table 2**). The model average of these four top models revealed that survival during the nestling period was positively correlated with an increased number of days post-hatch spent in the nest by the female (estimate: 1.08 ± 0.38 ; $p = 0.005$) and negatively correlated with an increased percentage Days T_{thresh} during the nestling period (estimate: -0.86 ± 0.37 ; $p = 0.021$; **Table 2** and **Supplementary Figure 5**). The effect of drought occurrence on survival during the nestling period was not significant in the averaged model (estimate: 0.71 ± 0.92 ; $p = 0.444$, **Table 2**), although 4 of 15 chicks (26.7%) that hatched in drought seasons fledged,

compared to 40 of 56 chicks (71.4%) in non-drought seasons (**Supplementary Figure 6**).

There were two top models identified for the effect of drought occurrence on female entry date, including the null model (model weights: 0.56 and 0.44 respectively; **Supplementary Table 2**), and on the number of days post-hatch spent in the nest by the female, also including the null model (model weights: 0.55 and 0.45 respectively; **Supplementary Table 2**). Model averages indicated that drought occurrence did not significantly affect the female entry date (estimate: -0.07 ± 0.11 ; $p = 0.552$) or the number of days post-hatch spent in the nest by the female (estimate: -0.03 ± 0.05 ; $p = 0.550$) for breeding attempts that hatched at least one chick (**Table 2**).

Thermal Conditions in Nest Boxes vs. Natural Cavities

A single top model was identified for T_{nest} , indicating that T_{nest} inside both nest boxes and natural cavities showed a significant

TABLE 2 | Bayesian generalized linear mixed model (BGLMer) of the outcomes of different stages [entire breeding attempt ($n = 88$), nest survival from female entry until hatch/fail ("pre-hatch period"; $n = 88$), and nest survival from hatch until fledge/fail ("nestling period"; $n = 88$)] of individual breeding attempts in the southern yellow-billed hornbills (*Tockus leucomelas*) as functions of female entry date, percentage Days T_{thresh} (days during the attempt on which T_{max} [mean maximum daily air temperature (T_{air})] > T_{thresh} ($T_{\text{air}} = 34.5^{\circ}\text{C}$)), drought occurrence over the breeding season (19 October–24 March), and the interaction between Days T_{thresh} and drought occurrence.

Response variable	Model family	Number of top models	Independent variables	Estimate $\pm$ SE (Adj SE for model averaged estimates)	Test statistic (z or t)	df	$p \leq$	Lower 95% CI	Upper 95% CI
Success (1) or failure (0) of the entire breeding attempt	Binomial	1	Female entry date	-1.10 ± 0.41	-2.72	86	0.007	-1.90	-0.31
			% Days T_{thresh}	-0.01 ± 0.58	-0.02	86	0.983	-1.15	1.12
			drought occurrence (non-drought)	1.54 ± 0.94	1.63	86	0.103	-0.31	3.39
			% Days T_{thresh} * drought occurrence	-2.65 ± 1.04	-2.54	86	0.011	-4.69	-0.61
			% Days T_{thresh} (drought seasons)	-0.20 ± 0.67	-0.29	21	0.771	-1.50	1.11
			% Days T_{thresh} (non-drought seasons)	-1.87 ± 0.65	-2.90	63	0.004	-3.14	-0.61
Survival (1) or failure (0) during the pre-hatch period	Binomial	1	Female entry date	-1.24 ± 0.44	-2.81	86	0.005	-2.10	-0.37
			% Days T_{thresh}	0.16 ± 0.52	0.32	86	0.751	-0.55	0.87
			Drought occurrence (non-drought)	2.97 ± 1.80	1.66	86	0.098	-0.85	1.17
			% Days T_{thresh} * drought occurrence	-1.55 ± 0.77	-2.00	86	0.045	-3.07	-0.03
			% Days T_{thresh} (drought seasons)	-0.91 ± 0.90	-1.02	21	0.310	-2.67	0.85
			% Days T_{thresh} (non-drought seasons)	-1.22 ± 0.00	-2.48	63	0.001	-2.160	-0.251
Survival (1) or failure (0) during the nestling period	Binomial	4	Female entry date	-0.11 ± 0.27	0.39	86	0.696	-1.09	0.44
			% Days T_{thresh}	-0.86 ± 0.37	2.30	86	0.021	-1.59	-0.13
			Drought occurrence (non-drought)	0.71 ± 0.92	0.77	86	0.444	-0.49	3.04
			Days post-hatch spent in the nest by female	1.08 ± 0.38	2.82	86	0.005	0.33	1.83
Female entry date	Poisson	2	Drought occurrence (non-drought)	-0.07 ± 0.11	0.59	86	0.552	-0.41	0.10
Days post-hatch spent in the nest by female	Poisson	2	Drought occurrence (non-drought)	0.03 ± 0.05	0.60	86	0.550	-0.05	0.19

Survival in the nestling period was also modeled as a function of the number of days post-hatch spent in the nest by the female.

BGLMer outcomes for female entry date and the number of days post-hatch spent in the nest by the female modeled as functions of drought occurrence also shown. Model outputs are for the single top model, or model averages when there was more than one competing model within 2 ΔAICc (see column "Number of top models"). All models contain the random factor "season."

For the categorical variable "Drought occurrence (drought/non-drought)," "drought" is set as the intercept.

Rows in italics show post hoc investigations of significant interactions between % Days T_{thresh} and drought occurrence.

Rows for independent variables with a significant effect are in bold.

The "*" symbolises an interaction between the two terms.

positive relationship with outside T_{air} (estimate: 1.03 ± 0.00 ; $p = 0.001$), but that there was a significant interaction between T_{air} and the type of nest (estimate: -0.49 ± 0.00 ; $p = 0.001$; **Supplementary Tables 3, 4**). The slope of the relationship between T_{nest} and T_{air} was $0.55 T_{\text{nest}}^{\circ}\text{C} \cdot T_{\text{air}}^{-1}$ for natural cavities (estimate: 0.55 ± 0.00 ; $p = 0.001$) compared to $1.03 T_{\text{nest}}^{\circ}\text{C} \cdot T_{\text{air}}^{-1}$ for nest boxes (estimate: 1.03 ± 0.00 ; $p = 0.001$), suggesting that T_{nest} within natural cavities was buffered against T_{air} , while T_{nest} within nest boxes tracked T_{air} (**Supplementary Table 4 and Supplementary Figure 7**). The difference between T_{nest} within nest boxes and natural cavities was most pronounced at higher T_{air} ; at $T_{\text{air}} > T_{\text{thresh}}$, the mean T_{nest} within nest boxes was $39.65^{\circ}\text{C} \pm 3.23$ SD, while the mean T_{nest} within natural cavities was $34.91^{\circ}\text{C} \pm 3.68$ SD (**Supplementary Figure 7**).

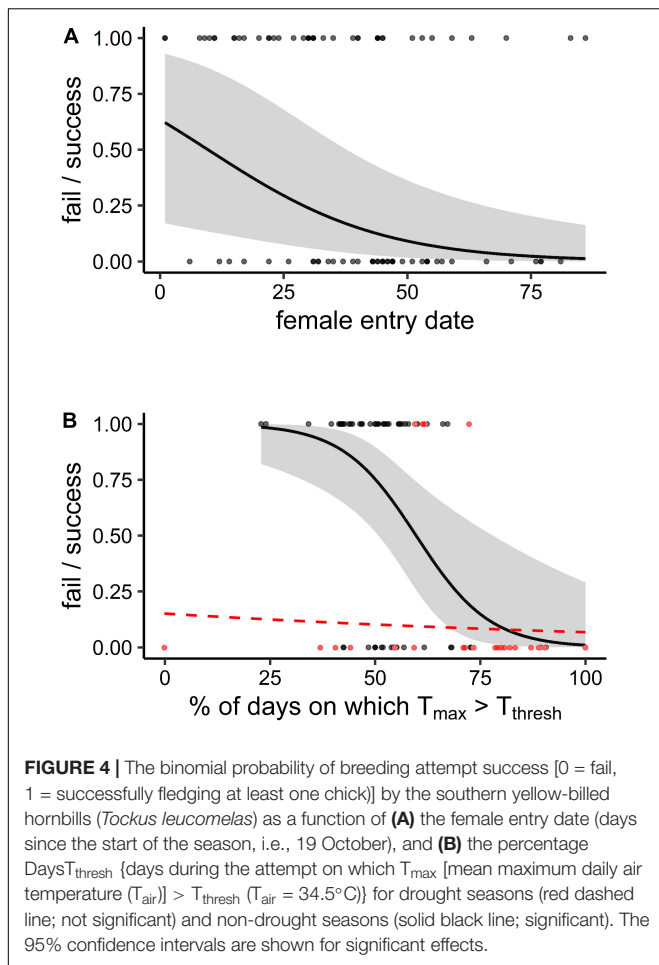
DISCUSSION

The negative impacts of extreme weather events and a rapidly warming climate on survival and reproduction are being recorded worldwide across multiple taxa (Moreno and Møller, 2011; Cunningham et al., 2021). Intensive monitoring allowed us

to quantify the breeding performance of our study population of hornbills over a decade. Breeding effort and performance of hornbills collapsed throughout our monitoring period, correlating to rapid climate warming in the region. Comparing the first three seasons (2008–2011) of monitoring to the last three (2016–2019), the percentage of boxes occupied declined from an average of 52–12%, the percentage of breeding attempts that succeeded from 58% to 17%, and the mean fledglings produced per attempt from 1.1 to 0.4. Breeding effort and performance were negatively correlated with both extrinsic (higher T_{air} and the occurrence of drought within the breeding season) and intrinsic (later dates of entry into the nest cavity and shorter time periods spent in the nest post-hatch by the female parent) factors. Given the severe negative effects of high T_{air} on both adult conditions (van de Ven et al., 2019) and breeding success (van de Ven et al., 2020a, this study) in this population of hornbills, we suggest that rapid warming has likely been the primary driver of their recent collapse in breeding success.

Climate and Hornbill Breeding

The decline in the percentage of nest boxes used over the decade of monitoring could be a result of fewer potential breeding pairs



in the study population as the monitoring period progressed (e.g., Rioux Paquette et al., 2014; Cruz-McDonnell and Wolf, 2016). However, the number of pairs present was continuously estimated to be approximately 20–25 each year, and in the majority of years since 2012, pairs were seen inspecting nest boxes between October and March but failed to ultimately breed (Pattinson and van de Ven, unpublished data). Moreover, there was a significant link between higher seasonal T_{air} and reduced nest box occupancy. Therefore, the declining breeding effort likely reflects an increasing number of resident pairs skipping breeding in response to increasingly challenging environmental conditions (i.e., higher T_{air} during the summer breeding season); skipping breeding is common in another southern African bucerotiforme, the southern ground-hornbill (*Bucorvus leadbeateri*; Carstens et al., 2019), and is suspected to occur in response to poor breeding conditions in various avian, mammalian, and amphibian taxa (e.g., Pietiainen, 1989; Pilastro et al., 2003; Kinkead and Otis, 2007; Keynan and Yosef, 2010; Cayuela et al., 2014; Griffen, 2018). Nesting hornbills experience low rates of predation because the birds seal up the entrance of the nest, leaving only a tiny slit through which food can be passed from the male to the female and chicks inside (Moreau and Moreau, 1941), so changes in predation rates with T_{air} or drought are unlikely responsible for variation in breeding performance.

Nest boxes were less buffered against changes in T_{air} compared to natural cavities. Therefore, our findings may represent a more severe response to high T_{air} than would be evident in birds breeding in natural cavities, especially given the strong effect of T_{nest} on fledging conditions (van de Ven et al., 2020a). However, the T_{air} recorded at our field site was consistently lower than that recorded by weather stations in surrounding areas, suggesting that the trends we measured are likely at least indicative of what is happening to populations of hornbills breeding in natural cavities in the hottest parts of their range. Moreover, variation in nest success is also driven by the effects of T_{air} on the provisioning behavior of the parents (van de Ven et al., 2019; van de Ven et al., 2020a) and drought, which are effects independent of nest type.

Inter-annual and within-season declines in breeding success were strongly associated with both high temperatures and drought. Of the 115 breeding attempts we recorded, none were successful when T_{max} exceeded $T_{\text{threshold}} \geq 72\%$ or more days during an attempt. The hottest period during which an attempt was successful involved 55 of 76 days (72%) with $T_{\text{max}} > T_{\text{threshold}}$, but all 18 attempts during hotter conditions failed, indicating the potential for the precipitous decline in breeding success at high T_{air} . Additionally, significantly more breeding attempts succeeded (fledged at least one chick) in non-drought seasons (61.5%) compared to drought seasons (17.4%). The near-ubiquitous nest failure across a range of T_{air} in drought years presumably occurred because low rainfall leads to low food abundance in arid and semi-arid systems since rainfall drives primary productivity and the energy available through the entire trophic cascade (Mares et al., 2017; Carstens et al., 2019). These results suggest that breeding performance in this population of hornbill is susceptible to both low rainfall and high T_{air} independently, compared to studies suggesting vulnerability only when low rainfall and high T_{air} co-occur (Flesch, 2014; Cruz-McDonnell and Wolf, 2016; Iknayan and Beissinger, 2018; Bourne et al., 2020b). Although our models did not indicate a statistically significant effect of drought during the nestling period, the much lower percent of nestlings that fledged in drought (26.7%, $n = 4$ out of 15) compared to non-drought (71.4%, $n = 40$ out of 56) seasons suggested that drought was associated with reduced nest success during both pre-hatch and nestling periods. In fact, the low number of chicks that hatch or fledge during drought seasons creates a limited sample size for analyses, likely reducing our power to statistically demonstrate the effect of drought.

Variation in breeding success within breeding seasons was also correlated to intrinsic factors including female entry date (when the female sealed herself in the nest box) and the number of days post-hatch the female was incarcerated in the nest. Later female entry date and fewer days post-hatch spent incarcerated by the female both correlated with lower breeding success. Delayed nest initiation is associated with drought and low resource availability in some species (McCreedy and van Riper, 2015; Carstens et al., 2019). However, rainfall in the 2 months preceding the breeding season had no significant effect on seasonal breeding output, and neither female entry dates nor the number of days post-hatch spent in the nest by the female parents were significantly related to the occurrence or absence of drought, suggesting that their effects were likely not artifacts of rainfall and food availability

(Harriman et al., 2017). Rather, we suspect that lower-quality parents delay the onset of breeding and/or that lower-quality females cannot stay incarcerated for extended periods. Fewer stored body reserves in either parent may preclude beginning breeding early or the ability to sustain continuous mass loss during the breeding attempt (van de Ven et al., 2020a). This pattern reflects widespread interactions between the timing of breeding and the quality of the parents (for review, see Verhulst and Nilsson, 2008) and supports the well-established concept of earlier breeding and higher parental quality positively affecting reproductive performance (Moreno et al., 2005; Verhulst and Nilsson, 2008; de Zwaan et al., 2019).

Our analyses of T_{air} and rainfall trends in the southern Kalahari revealed trends consistent with those reported in recent studies (Kruger and Sekele, 2013; van Wilgen et al., 2016; Mbokodo et al., 2020); T_{max} and $\text{Days}T_{\text{thresh}}$ during the breeding season of the hornbills have increased by more than 1.0°C and almost 20 days per decade, respectively, over the last ~ 25 years, whereas no long-term trend in rainfall/drought recurrence was apparent. This trend indicates that hornbills face severe challenges to their persistence across the seasonally hot, arid parts of their range. The negative effects of high temperatures will increasingly cause reduced breeding success even in non-drought years as global warming advances. Therefore, while the magnitude of population decline is limited by the severity of droughts and heatwaves, population recovery and persistence will be limited by a decreasing capacity of the hornbills to breed successfully in non-drought seasons due to increased mean T_{air} (Williams et al., 2016; Albright et al., 2017; Palmer et al., 2017; Conradie et al., 2019). Based on (1) the rapid rate of warming, (2) the fact that no breeding attempts succeeded if $\geq 72\%$ days during the attempt had $T_{\text{max}} > T_{\text{thresh}}$ (corresponding to a T_{max} during the attempt $\geq 35.7^{\circ}\text{C}$, a threshold which will be exceeded across the entire breeding season by ~ 2027 at our study site under current warming trends), and (3) 8 years is the longest a wild, color-ringed hornbill has survived in our study population, we arrive at the grim prediction that this population of hornbills could be extirpated by 2040. Moreover, model predictions suggest that the majority of the hornbill's range will approach the T_{air} threshold, above which breeding success is $< 50\%$ by the turn of the century (Conradie et al., 2019).

Conclusion

Many birds in seasonally hot, summer rainfall, arid zones are constrained to breed in response to, or to coincide with, rainfall, making it difficult for them to shift breeding dates outside of the hottest periods of the year which correspond with the rainfall season (Wolf, 2000; McCreedy and van Riper, 2015; Mares et al., 2017; Iknayan and Beissinger, 2018; van de Ven et al., 2020a). Therefore, even small increases in summer maximum T_{air} could drive large consequences during breeding (Sinervo et al., 2010; Alagaili et al., 2017; Albright et al., 2017; Riddell et al., 2019; McKechnie et al., 2021). Moreover, an increase in the frequency and severity of sub-lethal, suboptimal conditions that reduce parental quality may also reduce fledgling conditions, generally resulting in lower survival probability, recruitment, and lifetime fecundity (Dawson et al., 2005; Gardner et al., 2016;

Conradie et al., 2019; de Zwaan et al., 2019; Bourne et al., 2020a; Cunningham et al., 2021) (but see McLean et al., 2020). In the case of hornbills, there is evidence that the sub-lethal consequences of high T_{air} (regardless of high rainfall) and drought on the parents affect offspring quality (van de Ven et al., 2020a), and in this study, the probability of successfully fledging offspring or even attempting to breed at all. While the unusual breeding strategy of the hornbills could make them especially vulnerable, high temperatures and drought have negative impacts on breeding output in a variety of taxa across the globe (Welbergen et al., 2008; Sinervo et al., 2010; Gardner et al., 2014; Andreasson et al., 2020; van de Ven et al., 2020b; Cunningham et al., 2021). Therefore, we suggest that our findings are likely applicable to a range of species and support the proposition that even for species where catastrophic heat-related mortality events remain unlikely, climate change can drive rapid declines and potentially local extinctions (Conradie et al., 2019; McKechnie and Wolf, 2019; McKechnie et al., 2021). In fact, both Iknayan and Beissinger (2018) and Riddell et al. (2019) recently demonstrated that this extirpation process in arid zones is already taking place and is set to continue under future climate change.

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 Science Faculty Animal Ethics Committee, University of Cape Town, permit no. 2012/V44/PH, 2013/V24/PR, 2018/V15/SC, 2019/V6/SC; 2019/V16/SC, the Animal Ethics Committee of the University of Pretoria (protocol NAS 361/2019) and the Research Ethics and Scientific Committee of the South African National Biodiversity Institute (P19–23). The study was carried out on private land (Kuruman River Reserve, Leeupan Guest Farm and Rus en Vreede farm) with permission of the landowners and of the Northern Cape Department of Environment and Nature Conservation of South Africa (permit numbers 995/2012, 660/2013, 1166/2013; FAUNA 0172/2019).

AUTHOR CONTRIBUTIONS

AM, NP, SC, and TV developed the conceptual framework. LN, MF, NP, and TV collected data. NP analyzed the data and wrote the manuscript. All authors contributed to manuscript editing and 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.2022.842264/full#supplementary-material>

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Breathing in the Cold: Seasonal Changes in the Ventilatory Pattern in a Small Boreal Passerine Bird

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Small passerine birds in the north need to take advantage of several behavioral and physiological mechanisms to maintain energy balance during the winter characterized by low food supply, low ambient temperatures, and short days. Here we test if the breathing pattern of a non-migratory species, the great tit (*Parus major*), show seasonal variation that could help the species keeping a positive energy balance in the winter. To this aim, we measured oxygen consumption and ventilatory variables (tidal volume and respiratory frequency) in summer- and winter-acclimatized great tits exposed to ambient temperatures between -15 and 30°C . Winter-acclimatized great tits had a higher resting metabolic rate and a different breathing pattern compared to the summer-acclimatized birds. During the winter the great tits utilized a breathing pattern, consisting of an increased respiratory frequency to tidal volume ratio compared to summer-acclimatized birds at all temperatures. The higher oxygen uptake and the altered breathing pattern in the winter-acclimatized tits resulted in a higher lung oxygen extraction. However, during acute cold exposure neither the winter- nor summer-acclimatized great tits increased the oxygen extraction at low ambient temperature. The higher lung oxygen extraction in the winter-acclimatized tits implies that the birds will save on the minute ventilation, which reduces the evaporative water loss through respiration. The daily water loss saved can be more than 1 g of water per day. This is a substantial saving corresponding to a saving in evaporative heat loss corresponding to between 4 and 8% of the resting metabolic rate. This might be significant in keeping an energy balance, and the altered breathing pattern in the winter, ensuring an increased oxygen extraction, may therefore represents an additional physiological mechanism making it possible for small passerine birds to survive the northern winter.

Keywords: great tit (*Parus major*), lung oxygen extraction, metabolic rate, breathing pattern, seasonal acclimatization, thermoregulation

INTRODUCTION

Small passerine birds are, because of their high mass-specific metabolic rate and their high surface to volume ratio, especially vulnerable to environmental challenging conditions. Hence, those that are wintering at high latitudes, where they experience low ambient temperatures in combination with reduced food supply and only few hours of daylight, have evolved a suite of behavioral and physiological mechanisms to maintain an energetic balance (Irving, 1972;

Dawson and Marsh, 1989; Dawson and O'Connor, 1996; Reinertsen, 1996; Bodin, 2007; Olson et al., 2010; Swanson, 2010; Swanson and Vézina, 2015). These mechanisms enable small passerine birds to be year-round resident even at very high latitudes.

One of the most important behavioral adaptations in northern birds keeping an energy balance during the winter, is the ability to hoard food. This has been described in a series of classical papers by Haftorn (1954, 1956a,b,c) for several small northern passerine species. Also, the ability to forage at lower light intensities has also been described as a way of increasing the total time during a day that can be used for foraging. This has been demonstrated in the arctic living redpolls (*Carduelis flammea* and *C. hornemanni*; Brooks, 1968; Pohl, 1989). Other ways of ensuring an energy balance during cold winter nights is to store more body fat. Hence many passerine birds increase their fat reserves during the winter, with the amount of fattening being directly influenced by the prevailing ambient temperatures (Gosler, 2002; Krams et al., 2010).

Another way of ensuring an optimal economic use of the available energy during the northern winter is to use nightly hypothermia (Reinertsen and Haftorn, 1986; Reinertsen, 1996; Cooper and Gessaman, 2005; Bodin, 2007). This involves a temporal decrease of the body temperature, thereby decreasing the temperature gradient from the body core to the environment, resulting in a reduced heat loss. This has been most clearly shown in the Willow tit (*Poecile montanus*), where the nightly body temperature may decrease to values of 33°C, with the decrease being dependent on the ambient temperature (Reinertsen and Haftorn, 1986).

One of the main characteristics of winter acclimatization of small passerines from the temperate region is an increased metabolic capacity. Hence, small birds wintering in the north typically increase their maximum metabolic capacity during cold exposure in the winter, a feature that has been demonstrated to increase the probability of survival (Petit et al., 2017). The increased thermogenetic capacity might be caused by increased muscle mass, muscle structure or aerobic enzyme activity, which drive an upregulation of the metabolic machinery of the whole body (Swanson, 2010; Liknes and Swanson, 2011; Petit et al., 2014; Jimenez et al., 2019). Even the mitochondria of the red blood cells have recently been shown to be more thermogenic in the winter, further adding to an increased heat producing capacity in small avian species living in the north (Nord et al., 2021).

In addition to these behavioral and physiological mechanisms, the respiratory system has, apart from being directly responsible for the uptake of oxygen, also been advocated as playing a potential role in cold acclimatization in vertebrates (Mortola and Frappell, 2000). It is normally assumed that for most animals an increased metabolic rate during cold exposure is directly followed by a proportional increased in lung ventilation (Mortola and Maskrey, 2011). However, in some species the increase in ventilatory volume is less than the increase in metabolic rate, implying that less air is ventilated for a given amount of oxygen taken up by the lungs. Such a decrease in the ventilation-oxygen uptake relationship (often in the range of 5–15%; Mortola and Maskrey, 2011), suggest an increased “efficiency” of the lung

system during cold exposure, often expressed as an increased lung oxygen extraction (EO₂). Because the evaporative heat loss is inevitably correlated to the volume of lung air being expired, an increased EO₂ could be a mean of reducing the evaporative heat loss in the cold. Most studies investigating this have been conducted on birds experimentally exposed to decreased ambient temperatures, and mixed results have been reported. Although a few species actually increase EO₂ when experimentally cold exposed (Bech et al., 1984; Brent et al., 1984; Johannesen and Nicol, 1990), most do not (e.g., Bech et al., 1985; Clemens, 1988; Bucher and Morgan, 1989; Morgan et al., 1992; Bech and Nicol, 1999). This also led Morgan et al. (1992) to conclude, after studying species living in the Antarctic, that increasing EO₂, as a means of reducing ventilatory heat loss is not a general phenomenon in cold-adapted birds.

Although a changed lung efficiency may not play a large role during thermoregulating when acutely exposed to cold, it could still play a role on a more longer time scale such as seasonal acclimation to cold. Data to test this are, however, so far very scarce. Only two studies on birds have provided some evidence that there could be a seasonal effect on the ventilatory accommodation of oxygen uptake in the cold (Cooper and Same, 2000; Arens and Cooper, 2005b). It is therefore still an open question to what extent a change in lung ventilation play a role in the seasonal acclimatization of small northern birds, and no newer studies seem to have addressed this question.

The great tit (*Parus major*) is a stationary breeding species throughout most of Asia, North-West Africa, and Europe (Snow and Perrins, 1998). Being stationary throughout most of northern Europe, the species consequently experiences large seasonal changes in ambient temperatures, daylight, and availability of food. In the present study we asked the question, whether great tits, living close to the northern limit of their distribution, show seasonal changes in the ventilatory pattern and oxygen extraction, which may add to help keeping their energy balance. If changes in lung oxygen extraction is part of the physiological repertoire ensuring energy balance during the winter, we would expect to find increased values of EO₂ in the winter-acclimatized great tits.

MATERIALS AND METHODS

Experimental Animals and Housing Conditions

Birds used in the present study were all caught around Trondheim in mid Norway at 63°N. Average daily temperature in Trondheim is warmest in July with an average temperature of 14.8°C and coldest in February with an average of –1.2°C (Norwegian Centre for Climate Services [NCCS], 2021). However, temperatures down to –20°C during winter and up to 25°C in the summer are regularly recorded.

The summer-acclimatized (SA) individuals ($N = 6$) were captured and used during the period between 6 July and 16 September, while the winter-acclimatized (WA) individuals ($N = 5$) were caught and used between 30 November and 15 March. During captivity the birds were kept at ambient temperatures (T_a) and light-dark conditions which were close

to the average outdoor conditions for the season. Hence, in the winter they were normally exposed to a T_a around -5°C , while in the summer T_a was normally kept between 15 and 20°C . Each bird had its own private cage (see Reinertsen, 1982, for a detailed description) with free access to food and water. The birds were mainly fed sunflower seeds with occasionally mealworms provided in addition. During the winter, at sub-zero ambient temperatures, they were daily given fresh snow as a source of water. To reduce the effect of captivity, each individual bird was kept in captivity for a maximum of 2 weeks, during which time all measurements were obtained. After the experiments all birds were released at the site of capture.

Measurements of Oxygen Consumption

Metabolic rate was measured indirectly as rates of oxygen consumption obtained from birds resting in darkness during daytime, between 10:00 and 17:00 h. At the start of each run the birds were weighed and placed inside a 2.0 L metabolic chamber. The chamber had double walls through which an anti-freeze solution circulated, the temperature of which was controlled by a thermostat. An experimental run would last for 90–120 min, during which we normally would obtain values of VO_2 and ventilatory parameters (see below) during the last 30 min. We calculated 10-min running averages of VO_2 during the experiment, and the lowest value was used to represent the resting metabolic rate. The birds were not deprived of food prior to the metabolic measurements.

Oxygen consumption was measured by an open circuit respirometry system. Outside air was dried and pumped through the metabolic chamber (volume of 2,000 ml) at a constant flow of 250 ml/min. After removal of water vapor using silica-gel, a subsample of 100 ml/min of the excurrent air was directed through an O_2 analyzer (Applied Electrochemistry S-3A), which measured fractional O_2 content of the air. Reference measurements of incurrent air were taken at the beginning and at the end of each run. The actual air flow through the metabolic chamber was measured with a calibrated flowmeter (Flow control, model R-1, Applied Electrochemistry Inc., Sunnyvale, CA). The voltage output from the O_2 analyzer was recorded at intervals of 30 s by a data logger (Grant Squirrel; type 1,200). Rates of oxygen consumption (VO_2 ; ml O_2 /min) were calculated from the equation $\text{VO}_2 = V_{\text{Ex}} * (\text{FiO}_2 - \text{FeO}_2) / [1 - (1 - \text{RQ} * \text{FiO}_2)]$ (Lighton, 2008; Eq. 11.2), where FiO_2 and FeO_2 is the incurrent and excurrent oxygen fraction, respectively, and V_{Ex} is the measured excurrent air flow. The respiratory quotient (RQ) is assumed to be 0.8. Because of the relatively low airflow/volume ratio, FeO_2 was calculated according to the instantaneous method (Bartholomew et al., 1981; Lighton, 2008) and used for calculating VO_2 . When calculating values of mass-specific oxygen consumption, we used the mean body mass obtained from measurements of body mass immediately before and after an experimental run.

Ambient temperature in the metabolic chamber was measured to the nearest 0.1°C using a copper-constantan thermocouple positioned in the outlet port of the chamber and connected to the Squirrel data logger. VO_2 as well as ventilatory data were obtained at T_a 's between -15 and 30°C , guaranteeing

measurements at thermoneutral conditions assumed to be near 25°C (Broggi et al., 2005). Birds were allowed 2–3 days in captivity before being used, and they were thereafter measured randomly at different T_a 's during the stay in captivity. In addition, on each experimental day only a single T_a was used for a given individual. Most birds were measured twice at each T_a and the average values were used in the subsequent analyses.

Ventilatory Parameters

Simultaneously with the VO_2 measurements, recordings of ventilation were made using the barometric method (Drorbaugh and Fenn, 1955; Mortola and Frappell, 2013). A differential pressure transducer (Lighton, 1988) was used to measure pressure changes inside the metabolic chamber. A computer recorded the voltage output of the pressure transducer at intervals of 0.02 s in bouts of 30 s, and values of V_T and f were obtained from this period. During each run an injection of a known volume of air (0.50 ml) was used for calibration. The birds were always resting quietly and the oxygen uptake stable before any ventilation measurements were made. The mean amplitudes of the ventilation and calibration signals were used to obtain estimates of tidal volume (V_T , ml BTPS) according to the formula given by Stahel and Nicol (1988). Ventilatory frequency (f , min^{-1}) was obtained directly from the periodicity of the ventilation deflections. Minute volume (V_E , ml min^{-1} , expressed at BTPS conditions) was calculated as $V_T * f$. The oxygen extraction efficiency (EO_2 , %; i.e., the amount of oxygen taken up as a percentage of the oxygen present in the inspired air) was calculated using the formula $\text{EO}_2 = (\text{VO}_2 * 100) / (V_E * \text{FeO}_2)$, where VO_2 is the oxygen uptake expressed as ml O_2 /min, V_E is the minute ventilation expressed in STPD-conditions and FeO_2 is the excurrent oxygen fraction from the metabolic chamber.

As an expression of the breathing pattern (BP), we chose to use the ratio f/V_T (Milsom, 1989). Since the body mass changed significantly between seasons, and because tidal volume changes isometrically with body mass (de Souza et al., 2021), V_T was expressed as mass-specific values, as volume of air (expressed at BTPS conditions) per 100 g body mass. Consequently, the BP was calculated as $f/(V_T/100 \text{ g})$.

Body Temperature

Body temperature (T_b) was not measured during the metabolic experiments due to the stress this would impose on the birds. Consequently, in four birds from each season we measured body temperatures in separate experimental runs, which closely resembled the metabolic experiments except that the chamber was not completely sealed. This enabled us to remove the birds quickly and obtain a reading of T_b , usually within 60 s, using a Cu-Co-thermocouple inserted 10–15 mm into the rectum. Values of T_b were obtained in both WA and SA birds at four different ambient temperatures (-15 , 0, 25, and 30°C). The overall mean T_b obtained from WA and SA individuals were used in calculating V_T in WA and SA birds, respectively.

Calculations and Statistics

Each individual bird was often measured more than once at a given ambient temperature. Thus, for each individual bird a

mean value for each parameter at each T_a was calculated and used in all statistical tests. Values are presented as mean $\pm$ 1 SD. For comparison of mean values, a t -test was used. For analyses of the effect of season on the relationship between ambient temperature and VO_2 below thermoneutrality we used a one-way ANCOVA. Also, for analyzing whether season influenced the relationship between T_a and any respiratory parameter below thermoneutrality, we also use one-way ANCOVAs. In both cases body mass was included as a covariate and bird ID as a random factor. We used the same statistical approach when analyzing if season influenced how tidal volume or respiratory frequency influenced EO_2 . In the latter case data obtained at all temperatures between -15°C and 30°C were used. All statistical analyses were performed using IBM SPSS Statistics ver. 28 with the significance level set at 0.05, although we have adopted a more flexible use of this P -value threshold (Muff et al., 2022).

RESULTS

Body Mass

For each individual bird, an overall mean body mass was calculated. The mean body mass of the SA tits was 15.9 g ($SD = 1.3$ g, $N = 6$) and for the WA tits 17.9 g ($SD = 1.0$ g, $N = 5$). The body mass of the WA tits is higher than that of the SA tits (t -test, $P = 0.025$).

Body Temperature

Body temperatures were measured in four WA and four SA great tits. The T_b was not related ($P > 0.10$) to T_a in either summer or winter. However, the mean T_b of WA birds ($42.2 \pm 0.4^\circ\text{C}$) was significantly higher (t -test, $P < 0.001$) than the mean T_b of SA birds ($41.0 \pm 0.6^\circ\text{C}$).

Metabolic Rates

Within both SA and WA great tits there were no statistical differences between values of VO_2 obtained at ambient temperatures of 20, 25, and 30°C . However, the values obtained at T_a of 15 and at 20°C differed significantly ($P < 0.05$) within both seasons. Consequently, we assumed the values obtained between 20 and 30°C to represent thermoneutral values and the

mean oxygen consumption at these temperatures was used to represent the daytime resting metabolic rate (RMR). For WA tits thermoneutral VO_2 was 5.03 ± 0.56 ml O_2 g^{-1} h^{-1} and for the SA birds VO_2 was 4.17 ± 0.37 ml O_2 g^{-1} h^{-1} . There was strong statistical evidence ($P < 0.001$; **Table 1**) that thermoneutral RMR was higher in the WA tits than in the SA tits.

Below the thermoneutral zone the oxygen consumption increase linearly with decreasing ambient temperature for both groups of birds (**Figure 1A**). The relationships are best described by the equations: VO_2 (O_2 g^{-1} h^{-1} , summer) = $7.016 - 0.143T_a$ ($R^2 = 0.79$) and VO_2 (O_2 g^{-1} h^{-1} , winter) = $7.661 - 0.118T_a$ ($R^2 = 0.62$). There is only weak statistical evidence that the slopes of the lines differ (ANCOVA, $P = 0.051$).

Breathing During Thermoneutrality

Thermoneutral values of respiratory parameters are shown in **Table 1**. Remarkably, the mean values of tidal volume obtained in the two seasons were practically identical (**Table 1**). Our data on respiratory frequency, on the other hand, show evidence of higher values in the winter than in summer ($P < 0.01$; **Table 1**). Hence, the breathing pattern showed a noticeable seasonal change, with the BP being significantly higher in the winter, due to a change solely in the breathing frequency. Despite the increase in respiratory frequency in the winter, we found no evidence that minute ventilation changed with season ($P = 0.11$; **Table 1**). That the great tits do not show a change in total ventilation volume between seasons, and yet had a much higher oxygen uptake in the winter, are evidence of a higher lung oxygen extraction in the WA birds during thermoneutral conditions ($P < 0.05$; **Table 1**).

Breathing During Cold Exposure

When exposed to temperatures below the thermoneutral zone (TNZ), the total (minute) ventilation increased in both SA and WA birds due to an increase in both respiratory frequency and tidal volume (**Figures 1D–F**). However, the dependency of f or V_T in increasing the minute ventilation varied between the seasons. While the respiratory frequency was lower at all T_a 's in the summer (**Figure 1E**), the opposite was the case for tidal volume (**Figure 1D**; ANCOVA, $P < 0.001$ in both cases). Hence, in both seasons the increase in total ventilation was mainly due to an increase in tidal volume. As a result, the WA and the SA great tits follow distinct trajectories for the relationship between f and V_T when cold exposed (**Figure 2**). This also results in a clear difference in breathing pattern between season, with the WA great tits having a higher breathing pattern than the SA birds (**Figure 1B**). Using values for all tested ambient temperatures, season had a significant effect on the overall BP (ANCOVA; $P < 0.001$). There was strong evidence that T_a affected BP ($P = 0.004$) indicating a decrease in BP during cold exposure (**Figure 1B**). Season did not influence this decrease ($P = 0.91$ for the interaction season* T_a).

Lung Oxygen Extraction

The thermoneutral values of EO_2 differed significantly ($P = 0.029$) between SA and WA great tits, with a slightly higher mean lung oxygen extraction in the winter; 29.1 vs. 23.5% in the summer (**Table 1**). There was moderate evidence that EO_2

TABLE 1 | Mean values ($\pm$ 1 SD) of resting oxygen (VO_2) uptake and ventilatory parameters in summer- and winter-acclimatized great tits obtained at thermoneutrality, i.e., temperatures between 20 and 30°C (NS, non-significant).

	Summer	Winter	P^*
VO_2 (ml g^{-1} h^{-1})	4.17 ± 0.62	5.10 ± 0.59	<0.001
VT (ml, BTPS)	0.43 ± 0.09	0.43 ± 0.07	NS
f (min^{-1})	67.26 ± 10.09	78.99 ± 12.73	<0.01
BP [f /(VT/100 g)]	25.57 ± 6.18	33.63 ± 6.57	<0.01
VE (ml min^{-1} , BTPS)	28.84 ± 8.22	33.68 ± 8.41	NS
EO_2 (%)	23.47 ± 6.07	29.08 ± 7.82	<0.05

*Results of a Student's t -test.

V_T , tidal volume; f , respiratory frequency; VE , minute ventilation; BP, breathing pattern; EO_2 , lung oxygen extraction.

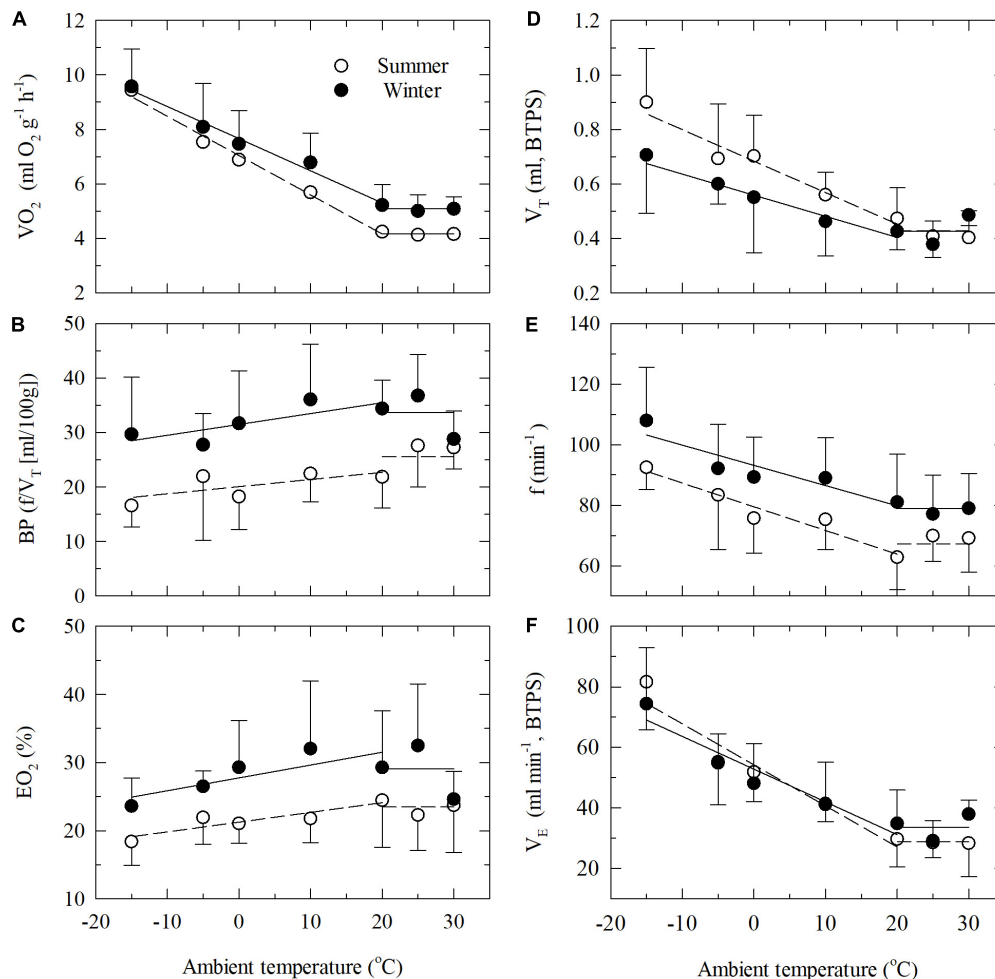


FIGURE 1 | Oxygen consumption (VO_2 , **A**) and ventilatory parameters [**B**) breathing pattern BP; **C**) lung oxygen extraction EO_2 ; **D**) tidal volume V_T ; **E**) respiratory frequency f ; and **F**) minute ventilation V_E] as functions of ambient temperature in summer-acclimatized (open symbols) and winter-acclimatized (closed symbols) great tits. Shown are mean values with one SD at selected ambient temperature. Mean thermoneutral values and linear regression lines below thermoneutrality are shown for summer-acclimatized (stippled lines) and winter-acclimatized (solid lines) birds.

decreased at lower ambient temperatures (ANCOVA; $P = 0.021$). However, the mean EO_2 during cold exposure is significantly higher (ANCOVA; $P < 0.001$, **Figure 1C**) during the winter than during the summer (estimates of 27.73 and 20.35, respectively). The seasonal change in EO_2 coincides with an overall change in breathing pattern, suggesting an association between the two variables. Indeed, there is very strong evidence of an overall relationship between BP and EO_2 (**Figure 3**, linear regression: $\text{EO}_2 = 13.23 + 0.42 \cdot \text{BP}$, $R^2 = 0.31$, $P < 0.001$). Within both seasons there was a significant effect of changes in V_T on EO_2 , with a decrease in lung oxygen extraction as tidal volume increased (**Figure 4A**). In both seasons the relationship between V_T and EO_2 was significant with the linear relationships being $\text{EO}_2 = 28.96 - 12.70 \cdot \text{V}_T$ ($R^2 = 0.26$, $P < 0.001$) for the summer and $\text{EO}_2 = 42.93 - 28.39 \cdot \text{V}_T$ ($R^2 = 0.40$, $P < 0.001$) for the winter. A significant interaction between season and V_T (ANCOVA, $P = 0.010$) points to V_T having different effects on EO_2 depending on season. The increase in EO_2 per unit decrease in tidal

volume is thus higher in the winter. Respiratory frequency also affects EO_2 significantly with a decrease in oxygen extraction as respiratory frequency increase (**Figure 4B**). Season does not influence this effect (ANCOVA; $P = 0.87$ for the interaction season $\cdot$ f). Overall, our results suggests that great tits during the winter, both at thermoneutrality and during cold exposure, have a higher lung oxygen extraction, caused by a seasonal change in their breathing pattern, with the winter-acclimatized tits breathing with a higher respiratory frequency to tidal volume ratio.

DISCUSSION

Thermoneutral Values

Our values of VO_2 and ventilatory parameters at thermoneutral conditions are all higher than expected based on allometric equations (de Souza et al., 2021). This is to be expected since

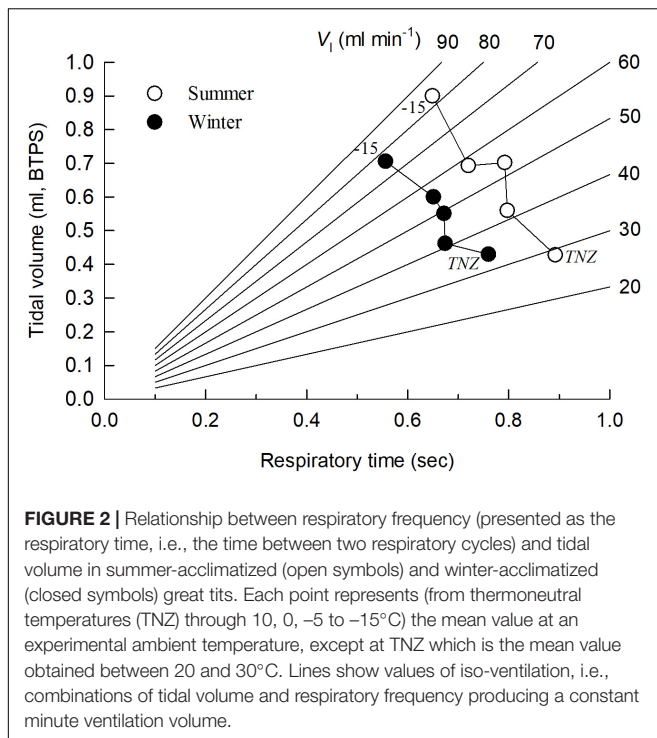


FIGURE 2 | Relationship between respiratory frequency (presented as the respiratory time, i.e., the time between two respiratory cycles) and tidal volume in summer-acclimatized (open symbols) and winter-acclimatized (closed symbols) great tits. Each point represents (from thermoneutral temperatures (TNZ) through 10, 0, -5 to -15°C) the mean value at an experimental ambient temperature, except at TNZ which is the mean value obtained between 20 and 30°C. Lines show values of iso-ventilation, i.e., combinations of tidal volume and respiratory frequency producing a constant minute ventilation volume.

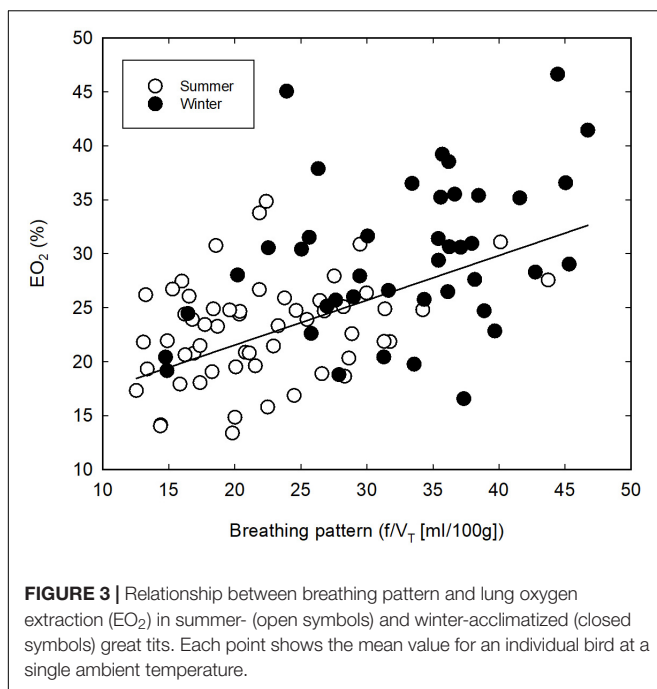


FIGURE 3 | Relationship between breathing pattern and lung oxygen extraction (EO_2) in summer- (open symbols) and winter-acclimatized (closed symbols) great tits. Each point shows the mean value for an individual bird at a single ambient temperature.

our birds were measured during daytime and were not in a postabsorptive phase, while allometric equations are based on “BMR-conditions.” Our values of thermoneutral VO_2 are 55 and 22% higher than the expected BMR for winter- and summer-acclimatized tits, respectively. Likewise, all ventilatory parameters are similarly higher than expected, varying between only 1% (summer breathing frequency) and 42% (summer tidal

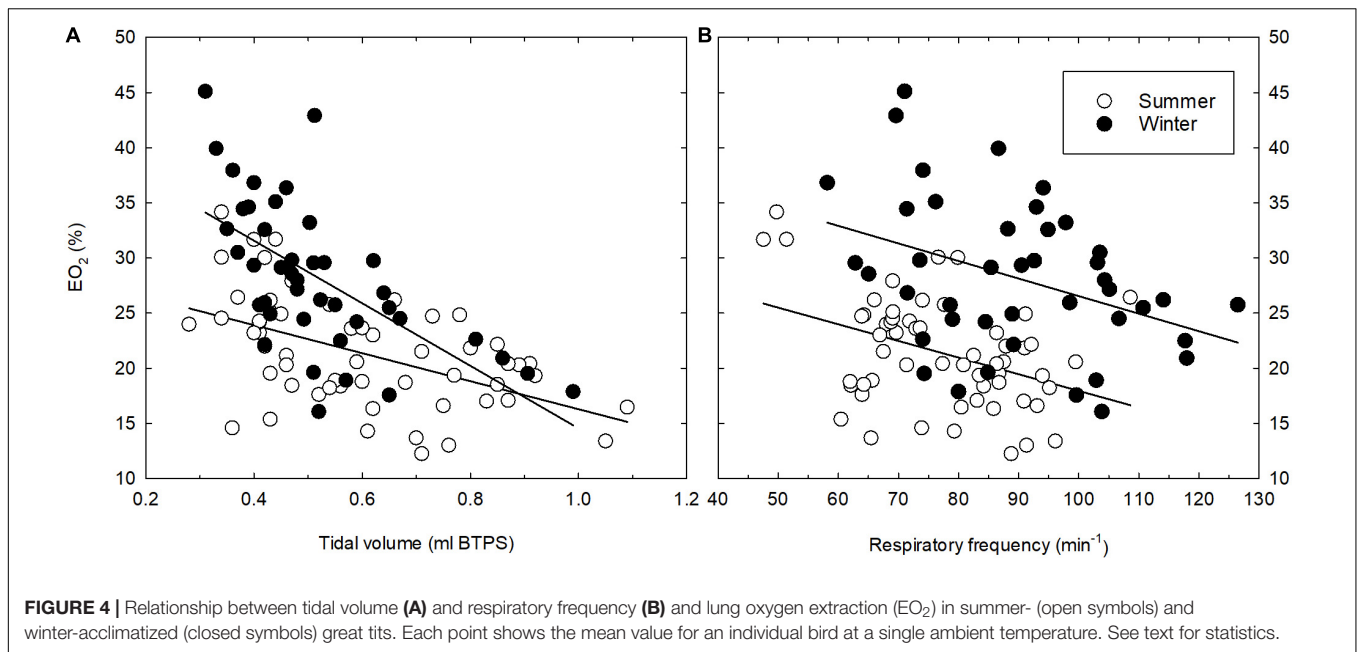
volume). However, as all birds were treated alike during the experimental procedures, the relative changes described here should still be representative for what would be the natural response. In addition, the condition with a postabsorptive phase and thermoneutrality is rarely experienced by wild great tits, who during normal activity are more likely to experience conditions like that created during the experimental runs. Anyway, our results on VO_2 conform to a general trend among high latitude birds in which there is a seasonal difference in BMR with a higher value during the winter (Swanson, 2010; McKechnie et al., 2015). Specifically in great tits, studies have demonstrated a higher metabolic rate in winter than in summer (e.g., Broggi et al., 2019). A higher resting metabolic rate in winter may indicate a need for higher metabolic capacity during the coldest months of the year (McKechnie et al., 2015).

Breathing in the Cold

The increase in minute ventilation with decreasing ambient temperature is a result of a simultaneous increase in both V_T and f (Figures 1, 2), with a larger contribution from tidal volume in both seasons. This is similar to the results in two species of finches (Rosy finches *Leucosticte arctoa* and House finches *Carpodacus mexicanus*) studied during cold exposure (Clemens, 1988), as well as in many other bird species (Bernstein and Schmidt-Nielsen, 1974; Bucher, 1985; Kaiser and Bucher, 1985; Bech and Nicol, 1999). However, a few species mainly increase respiratory frequency in the cold (e.g., Bucher, 1981).

During acute cold exposure, the lung oxygen extraction of the great tits did not change, which are in accord with most other studies on the effect of cold exposure in birds (Bech et al., 1985; Clemens, 1988; Bucher and Morgan, 1989; Morgan et al., 1992; Bech and Nicol, 1999). This is also in line with most mammalian studies in which the accommodation of the increased oxygen uptake at low ambient temperatures are being met by ventilatory response rather than a change in EO_2 (e.g., Chappell, 1992; Warnecke et al., 2010), although a few species increase EO_2 in the cold (e.g., Chappell and Roverud, 1990).

Acclimatization to winter conditions, in contrast, elicited a generally higher lung oxygen extraction in the great tits irrespective of ambient temperatures. There are only two studies to which these results can be compared. In the house sparrow (*Passer domesticus*) exposed to low ambient temperatures Arens and Cooper (2005b) also reported a higher EO_2 in winter-acclimatized individuals compared to summer-acclimatized individuals. The difference, however, was only observed during night-time. A stable EO_2 regardless of season during BMR-conditions was confirmed in another study on the same species by the same authors (Arens and Cooper, 2005a). In another small passerine bird, the black-capped chickadee (*Poephila atricapillus*), winter-acclimatized individuals are also reported to have a significant higher EO_2 during acute cold exposure compared to summer-acclimatized individuals (Cooper and Same, 2000), although EO_2 at BMR-conditions apparently also did not differ between seasons. Our study therefore supports these few other studies in the demonstration of a higher EO_2 during the winter compared to summer even at thermoneutral conditions.



Our results have demonstrated strong evidence of a negative association between tidal volume and lung oxygen extraction (Figure 4A). A similar “strong inverse correlation between EO_2 and V_T ” was also described by Clemens (1988) for the two species of finches. Obviously, if tidal volume should be so small that the dead space contributes a large fraction of V_T , EO_2 would necessarily decrease. This indicates that there should be an optimal value of V_T generating the highest oxygen extraction. Bucher (1985) advocated an interesting explanation for this, based on the assumptions that the bird lung has a constant volume with pulsative airflow (Maina, 2015; Powel, 2015). Bucher (1985) argued that an increase in V_T above an optimal size would move the amount of air equal to the increase in V_T past the gas exchanging tissue. The extraction of oxygen from this extra amount of air should therefore not be optimal. Under these circumstances, there should be a decrease in EO_2 as the V_T increases above the optimal size. This model was later challenged by Clemens (1988) who argued that the air flow through the avian parabronchial lungs was mainly continuous and not pulsative. More detailed studies on the avian lungs have demonstrated more complex structure with a “new” lung, the neopulmo, where air flow is indeed bidirectional, while being unidirectional in the “older” parabronchial parts of the lung (Maina, 2015; Powel, 2015). The distribution of the increased amount of inspired air within the avian lung with increased V_T is still not known. Such detailed information could possibly explain the relationship between tidal volume and lung oxygen extraction. Also, one can only speculate on why the great tits are not utilizing another breathing pattern and consequently an increased lung oxygen extraction also in the summer. We can offer no answer to this. However, it is interesting that in a broader context, i.e., moving taxonomically from low metabolic animals to high metabolic animals the BP has been shown to increase in parallel to the increase in total ventilation (Milsom, 1989). Our

results demonstrate that the same can be the case intraspecifically when species adapt to seasonal environmental changes.

How much would then the described seasonal change in breathing pattern, with a resulting increase in EO_2 , mean in saved evaporative water loss? Since the overall (both within and below TNZ) winter EO_2 is 28.2% and summer EO_2 is 21.4%, one can calculate the saving in volume of expired air in the winter, as the extra minute ventilation needed for uptake of the winter VO_2 at -15°C (2.85 mlO_2/min) assuming an EO_2 of only 21.4%. Using the equation for EO_2 we arrive at a saving of 19 ml/min . Using 51.1 mg/L as the saturated water content at a body temperature of 40°C (Hill et al., 2016), the saving of evaporative water loss (EWL) adds up to 57 $\text{mg H}_2\text{O}/\text{h}$, which mean a potential saving of more than 1 g of water (body mass) per day. This is a substantial saving, considering that great tits may lose up to 2 g of body mass during a single night (Bednekoff et al., 1994). Using the value for the latent heat of vaporization at 40°C of 2.41 $\text{kJ}/\text{g H}_2\text{O}$ (Withers, 1992) the saved heat loss due to the reduced EWL amounts to 0.137 kJ/h , corresponding to 8% of our measured metabolic rate at thermoneutrality, or 4% of the metabolic rate at -15°C in winter-acclimatized great tits.

Although great tits may use mechanisms that alleviate their nightly energy loss through ventilation, such as lowering their body temperature (Reinertsen and Haftorn, 1986) and using overnight quarters with more suitable ambient temperatures (Velký et al., 2010), our calculation still demonstrate that increasing the efficiency of the lungs in winter could be of potential importance in keeping energy balance in the winter. Hence, together with food hoarding, the storing of fat, hypothermia, and a changed aerobic machinery, the altered breathing pattern, with a resultant change in oxygen extraction, may thus represents an additional physiological mechanism making it possible for small passerine birds to live through the cold and dark northern winter.

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 and the experimental conditions for birds at the department was reviewed and approved by the Norwegian Animal Research Authority (Permit number S-0028/01).

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AUTHOR CONTRIBUTIONS

CM and CB designed and performed the experiments. CM wrote the first draft of the manuscript. CB wrote the final version with contributions from CM. Both authors contributed to the article and approved the submitted version.

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