Adaptive developmental plasticity and hormetic effects in the zebra finch (*Taeniopygia castanotis*)

by

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Abstract

Stressors experienced during development can have persisting detrimental effects on an individual's phenotype, as well as indirect effects on the phenotype of offspring. However, developmental stressors can also be beneficial depending on the timing, intensity, and duration of the stressor. Stressor exposure during development may act instructively, inducing changes in phenotype that make the organism, and sometimes their offspring, more suited for the potential poor environmental conditions they will experience in adulthood. This is known as adaptive developmental plasticity. Although it has the potential to influence population dynamics and evolutionary change, research examining adaptive developmental plasticity in endothermic vertebrates such as birds is limited. Moreso, we have a poor understanding of the underlying physiological changes mediating these effects, the potential trade-offs with key fitness-related traits, and how these relationships vary due to the adult environment. I address these gaps through experimental studies in the laboratory utilizing zebra finches (*Taeniopygia castanotis*). We exposed the finches to a prolonged mild heat (38 °C) conditioning or control (22 °C) treatment during juvenile development. As adults, the finches were exposed to a high heat stressor (42 °C) or control (22 °C) treatment in a 2x2 factorial design. We measured multiple cellular and life-history traits to determine 1) whether exposure to the mild heat conditioning during development induces an adaptive phenotypic adjustment, giving the finches an increased ability to buffer the negative effects of the high heat stressor as adults, 2) what physiological changes mediate this potential adaptive plasticity, 3) the potential costs and trade-offs between traits associated with the phenotypic change in stressful and benign environments. Furthermore, we determined whether the mild heat conditioning induced adaptive intergenerational plasticity, preparing the offspring of the finches to better cope with elevated ambient temperatures.

In Chapter 2, we saw that exposure to the mild heat conditioning induced a hormetic effect, preventing an increase in egg laying latency in adulthood after exposure to the high heat stressor. However, we also saw that exposure to either the conditioning as juveniles and/or the high heat stressor as adults had a stimulatory effect on the clutch viability of female finches. Surprisingly, there was seemingly no negative impacts of the high heat stressor on immune function (measured via wound healing) or several female reproductive parameters. However, there seemed to be a potential cost of the phenotypic change induced by the mild heat conditioning, but only when their adult environment did not "match". The female finches exposed to the mild heat conditioning as juveniles and the control treatment as adults had wounds that took longer to heal than those exposed to the high heat stressor as adults, regardless of whether they were exposed to the conditioning during development. These results indicate the potential for the presence of trade-offs between traits or a shift in life-history strategy.

In Chapters 3 and 4, we investigated whether exposure to the mild heat conditioning during development resulted in adaptive variation in adult antioxidant enzymes and heat-shock protein expression, reducing oxidative damage when exposed to the high heat stressor as adults. Moreso, we examined the relationships between the oxidative measurements and beak color - a sexually selected trait - to determine if the physiological changes induced trade-offs with, or buffered negative effects of heat stress on beak color. We saw that conditioned males had higher beak saturation and lower brightness in adulthood, but the response to the high heat stressor varied. When exposed to the high heat stressor as adults, conditioned males had higher levels of superoxide dismutase 1 & 2 antioxidant enzymes and reduced levels of HSP90 and HSP60 in the testes, as well as lower levels of cellular lipid oxidative damage in the liver, indicating an adaptive phenotypic change.

In Chapter 5, we investigated whether altered responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis resulting from the mild heat conditioning acts as a mediator of adaptive developmental plasticity, and the association with DNA damage and survival when exposed to the high heat stressor as adults. Conditioned female finches had higher baseline DNA damage levels and body masses than juvenile control females, as well as lower corticosterone levels following the adult treatment. However, once again we saw a cost when there was a mismatch between the juvenile and adult environment, as conditioned females exhibited reduced survival if they were not also exposed to the high heat stressor as adults.

In Chapter 6, we investigated whether maternal exposure to the mild heat conditioning during development would induce a form of intergenerational phenotypic plasticity known as maternal effects, and how the direction of this effect would be influenced by the maternal adult environment. Moreso, we determined whether the conditioning would act as an anticipatory maternal effect, resulting in offspring that are better suited to cope with high incubation temperatures as embryos. We found that embryos from conditioned mothers exhibited reduced water loss, longer development times, and heavier pectoralis muscles as hatchlings, when incubated at high incubation temperatures, compared to offspring from control mothers. The conditioned mothers that were also exposed to the high heat stressor as adults laid eggs with a higher density of shell pores, and embryos with lower heart rates during development. However, embryos from these conditioned and heat stressed mothers had reduced survival at control incubation temperatures, once again indicating potential costs when the future environment does not “match” the environment which induced the phenotypic change.

The chapters of this dissertation demonstrate that mild developmental stressors can have beneficial effects on phenotype in zebra finches, however, the effects are clearly context

dependent. The adaptiveness depends on both the trait being measured and the matching between the early life and adult environment of the finches. Mild developmental stressors can clearly have persisting impacts on a fitness-related traits at multiple biological levels, and research should integrate this knowledge when determining how populations will respond to rapid environmental change.

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List of Abbreviations

DPH	Days post-hatch
ED	Embryonic day
LMM	Linear mixed effects model
COXME	Mixed-effects Cox proportional hazards model
CI	Confidence intervals
GLMM	Generalized linear mixed-effects model
SD	Standard deviation
ROS	Reactive oxygen species
SOD	Superoxide dismutase
GPx	Glutathione peroxidase 1
4-HNE	4-hydroxy-2-nonenal
CORT	Corticosterone
HSP	Heat-shock protein
HPA-axis	hypothalamic-pituitary-adrenal axis
ELISA	enzyme-linked immunosorbent assay
RADD	Repair Assisted Damage Detection
MR, GR	mineralocorticoid and glucocorticoid receptors

Chapter 1: General Introduction

As a result of anthropogenic climate change, average ambient temperatures and the magnitude of extreme weather events are increasing (Smith et al., 2014; Ummenhofer and Meehl, 2017; Stillman, 2019). These increases can have massive impacts on ecosystems through mass mortality events, species range shifts, and extinction (Pounds et al., 1999; Thomas et al., 2006; Parmesan, 2006; Fey et al., 2015; Ratnayake et al., 2019). Populations with slower growth rates are thought to potentially be at a greater risk of extinction due to longer generation times, reducing the speed at which genetic adaptation to the changing temperatures can occur (Chevin et al., 2010). However, besides dispersal and genetic adaptation, populations may persist in the face of rapid environmental change through phenotypic plasticity - the ability of one genotype to produce multiple phenotypes (Davis et al., 2005; Chevin et al., 2010; Chevin and Lande, 2010). Therefore, understanding the underlying physiological plasticity in response to rapidly changing ambient temperatures is essential in predicting how different species and populations will respond in the face of climate change.

Developmental plasticity

Your sweat glands and choice of romantic partner as an adult have (hopefully only) one thing in common. They are both influenced by your developmental environment (Kawahata and Sakamoto, 1951; Conger et al., 2000; Simmons et al., 2014). The conditions an individual experiences during development have been well documented for their ability to have persisting effects on physiology, behavior, survival, and reproduction (Birkhead et al., 1999; Naguib et al., 2006; Monaghan, 2008). As such, developmental plasticity – environmentally induced alterations in developmental trajectories that create variation in the adult phenotype – have been

of particular interest in the study of adaptive phenotypic changes. Developmental plasticity has been demonstrated to be induced by a wide range of environmental factors such as diet, predation, competition, and thermal stress (Moe et al., 2004; Benard, 2004; Costantini et al., 2012; O'Hagan et al., 2015). Moreso, phenotypic plasticity induced during development can influence the phenotype of the subsequent generations without affecting the DNA, often known as transgenerational plasticity or parental effects (Mousseau and Fox, 1998; Cavieres et al., 2019; Hoffman et al., 2023). Due to the potential impact on phenotypes and fitness both within and across generations, developmental plasticity may significantly influence population dynamics and evolutionary change (West-Eberhard, 2005; Prudic et al., 2011; Badyaev, 2014).

Developmental stress

The influence of the developmental environment on phenotypic plasticity has been often studied in the context of stressful conditions, such as experimentally elevated glucocorticoid hormones, nutritional stress, or elevated ambient temperatures (Love and Williams, 2008; DuRant et al., 2013; Noguera et al., 2015; Wada and Coutts, 2021). Exposure to stressors during development was classically seen as unfavorable due to well documented negative effects in numerous taxa. Perhaps the ultimate boogeyman was exposure to chronic stressors during development, which can have detrimental effects on important traits such as growth, immune function, reproduction, cognitive function, and longevity (Cyr and Romero, 2007; Lupien et al., 2009; Monaghan et al., 2012; Chin et al., 2013; Crino et al., 2014). However, developmental stressors, even those that are chronic, have been recognized for their ability to have beneficial effects on fitness, including in birds (*reviewed in* Crino and Breuner, 2015). For example, male zebra finches (*Taeniopygia castanotis*) that had experimentally elevated corticosterone levels as

nestlings sired a higher number of offspring than control males (Crino et al., 2014). So then, what determines whether a developmental stressor will be detrimental or beneficial? A large part of the equation lies in the context of the individuals' later life environmental conditions.

Adaptive developmental plasticity

There is an (often misattributed) quote about judging a fish by its ability to climb a tree. I think it is too cliché to have the quote in my dissertation, but the general principle of evaluating performance in an improper context is applicable to the study of developmental stress. The long-standing view of developmental stress as wholly detrimental was in part due to the ways in which the resulting effects were evaluated. The potential beneficial effects of developmental stressors can occur at different time points and life-history stages. While developmental plasticity can induce changes that help an individual cope with its current conditions, in many scenarios the benefits may be delayed (Kruijt and Meeuwissen, 1991; Metcalfe and Monaghan, 2001). Much of the adaptive potential lies in the ability for developmental stressors to act instructively, resulting in phenotypic adjustments that make the organism more suited for the environment they are likely to experience in adulthood (Gluckman et al., 2005; Monaghan, 2008). One of the underlying principles of such "predictive adaptive responses" is that the environmental cues which induced the phenotypic change are somewhat predictive of the future environment (Bateson, 2001; Gluckman et al., 2005; Bateson et al., 2014). If the individual's lifetime environment is not relatively stable, then their adult environment and the developmental environment which induced the phenotypic change may not "match", resulting in maladaptive rather than adaptive plasticity (Gluckman et al., 2005). This acts as the basis of the "environmental matching" hypothesis (Monaghan, 2008). The effects of developmental stressors

should then be evaluated in multiple environmental conditions, as it can give you information as to the costs and adaptiveness of the adjustment which will influence the potential for selection. Evaluation of developmental plasticity in later environmental conditions similar to the early environment which caused the phenotypic change can provide information on its adaptive value, whereas evaluation in non-stressful conditions may provide information on underlying costs when the change is maladaptive (Gluckman et al., 2005; Monaghan, 2008; Taborsky, 2017). This makes fully factorial experimental designs and multiple sampling points essential in examining the potential adaptive effects of developmental stressors, and the lack of such design in past experimental studies may contribute to the absence of beneficial effects observed (Uller et al., 2013; Groothius and Taborsky, 2015).

Factors influencing the effects of developmental stress

Aside from the context of the adult environment, other factors can influence whether developmental stressors will be adaptive or maladaptive and whether the effects will be transient or long-lasting. These factors include the timing, duration, and intensity of the environmental cues (Scheiner et al., 2013; Eyck et al., 2019). As discussed, the developmental period has been well established as an important ontogenetic period for generating adaptive plasticity both within and across generations. However, even within development there may be important temporal windows which influence the strength and direction of a stressor's effects on phenotype (Hoverman and Relyea, 2007; Fawcett and Frankenhuis, 2015; Eyck et al., 2019). Pre- vs post-natal exposure to stressors can exert very different effects. For example, in mammals, offspring exposed to stressors pre-natally generally exhibit hypothalamic–pituitary–adrenal axis (HPA axis) hyper-responsiveness, while post-natal exposure can result in attenuated responsiveness

later in life (Henry et al., 1994; Liu et al., 1997; Macrì et al., 2004; Kapoor et al., 2006). Such variation between pre- and post-natal exposure has also been demonstrated in birds, which have neuroendocrine systems extremely similar to mammals (Wingfield, 2005; Love and Williams, 2008; Marasco et al., 2012; Marasco et al, 2016). While the differential effects are likely due to variation in sensitivity to environmental cues at different points in development, the windows for inducing adaptive developmental plasticity are not well understood (Hoverman and Relyea, 2007; Fawcett and Frankenhuis, 2015; Eyck et al., 2019).

The duration of developmental stressors also plays a significant role in their effects on phenotype both within and across generations, and the interaction between developmental stressor and ontogenetic timing may be the best predictor of the strength of effects on phenotype (Eyck et al., 2019). Generally, stressors of longer duration correspond with stronger long-term effects on phenotype (Swain and Lindsey, 1986; Salinas and Munch, 2012; Dupont et al., 2013; Suckling et al, 2014a; Suckling et al., 2014b). It is believed the increased effect sizes of developmental stressors due to increased stressor duration are the result of proportional alterations to developmental processes (O'Keeffe and Baum, 1990; Herbert and Cohen, 1993). Therefore, developmental stressors of longer duration may be more likely to produce adaptive effects, because the magnitude of the stressor is informative of its reliability as a predictor of future conditions (Sheriff and Love, 2013). If an environmental stressor experienced is chronic, it may suggest that the change in conditions will persist and also be experienced later in life as well as by the offspring, strongly necessitating a phenotypic change to maintain fitness (Sheriff and Love, 2013). On the other hand, an environmental stressor of short duration could be unpredictable and a poor indicator of future conditions, and less likely to induce long-term phenotypic change due to increased risk of environment-phenotype mismatch (Bonamour et al.,

2019). For example, sheepshead minnows exposed to 24 °C and 34 °C produced offspring with increased growth at those temperatures, however, this parental effect was only seen when the parents were exposed for 30 days compared to 7 days (Salinas and Munch, 2012).

Hormetic effects

Another factor that influences the adaptive potential of developmental stressors is the intensity of the environmental cue. While stressors are widely recognized for their ability to negatively impact an organism, mild levels of a stressor can have stimulatory and beneficial effect on traits. Such dose-dependent response is known as hormesis (Mattson and Calabrese, 2010). Hormesis, in the classical definition, describes a biphasic or inverted U-shaped curve of a trait in response to a stressor or toxicant (Mattson and Calabrese, 2010). In essence, mild levels of a toxin/stressor have a beneficial effect whereas high levels of that toxin/stressor become detrimental (Costantini et al., 2010; Mattson & Calabrese, 2010). Studies in the fields of toxicology and human health show that such nonlinear dose-response relationships result from a multitude of different toxicants and stressors. For example, *Drosophila* exposed to low levels of ethanol exhibit increased longevity, whereas ethanol exposure at high levels results in a shortened lifespan (Holmes et al., 1980). The term hormesis has also been used in a second, yet slightly different meaning. Sometimes referred to as "preconditioning" or "conditioning hormesis", the second use of the term describes a phenomenon where exposure to a mild stressor results in a phenotypic adjustment that allows an organism to cope with a higher severity of the stressor later in life (Calabrese et al., 2007; Mattson and Calabrese, 2010). Conditioning hormesis is therefore a form of adaptive phenotypic

"The dose makes the poison"
-Paracelsus

plasticity (or predictive adaptive response) that essentially gives the organism a persisting increased tolerance to a stressor, and there is evidence that it is stronger when the initial exposure occurs during development (Bartling et al., 2003; Honma et al., 2003; Le Bourg et al., 2004; Calabrese et al., 2007). For example, when juvenile zebra finches (*Taeniopygia castanotis*) were exposed to a mild heat conditioning (38 °C) as juveniles they suffered less plasma oxidative damage when exposed to a high heat stressor (42 °C) in adulthood, compared to juvenile controls (Costantini et al., 2012). Exposure to the mild heat conditioning also did not cause an increase in oxidative damage as juveniles. As predicted, the beneficial effect of the juvenile thermal conditioning was also dose dependent. The finches exposed to a higher magnitude (42 °C) thermal conditioning rather than the mild heat as juveniles did not exhibit tolerance to the high heat stressor (42 °C) in adulthood, suffering increased plasma oxidative damage relative to the mild heat conditioned juveniles (Costantini et al., 2012).

The beneficial effect of mild stressors also has the potential to carry over to the next generation. Adaptive within-generation plasticity can also result in parents producing offspring with phenotypes that increase fitness in the environment they will encounter, as parental phenotypic change in response to environmental cues are often more likely to provide accurate predictors of future conditions (Kuijper and Hoyle, 2015; Leimar and McNamara, 2015). While adaptive developmental plasticity may very likely influence how populations and species respond to rapidly changing climate conditions, most research utilizes invertebrates as a study species and measure a limited range of response traits when evaluating the adaptiveness of the phenotypic change. Consequently, we still have an incomplete understanding of the physiological changes underlying such adaptive developmental plasticity (Costantini et al., 2010).

Mechanisms underlying adaptive developmental plasticity

One of the largest outstanding questions in the study of adaptive developmental stressors and hormetic effects is regarding what physiological mechanisms act as mediators between environmental cues and variation in developmental processes. The answer likely lies in the physiology of the stress response itself. When organisms are confronted with unpredictable environmental conditions, the result is a host of physiological, behavioral, and molecular changes that promote the immediate survival of individuals and their return to homeostasis (Wingfield et al., 1998; Sapolsky et al., 2000). This is often known as the stress response. Developmental stressors have been demonstrated to have persisting effects on many of the endogenous physiological systems involved in the stress response, such as hormone secretion, antioxidant defense mechanisms, and heat-shock protein synthesis (Spencer et al, 2009; Costantini, 2014; Noguera et al., 2015; Kang and Shim, 2021; Madkour et al., 2021). The endocrine system has been of particular interest as potential mediator of these adaptive developmental adjustments, as hormones act as a link between environmental conditions and the genome and have well documented organizational effects on phenotypic development (Dufty et al., 2002; Lema and Kitano, 2013). Glucocorticoid hormones such as corticosterone (CORT), which is under control of the hypothalamic-pituitary-adrenal (HPA) axis, play an integral role in the stress response (Sapolsky, 1992). While glucocorticoids have a range of effects on both behavior and physiology at baseline levels that regulate metabolism and homeostasis, the rapid increase in response to a stressor promotes short term survival and recovery while also suppressing important processes such as growth and reproduction (McEwen and Wingfield, 2003; Romero and Butler, 2007). Therefore, glucocorticoid hormones act as an important mediator of life-history traits and trade-offs (Sapolsky et al., 2000; Romero, 2004).

Persisting changes in glucocorticoid secretion have been deemed as a mediator of adaptive developmental plasticity and stress tolerance (Gluckman et al., 2005; Zimmer et al., 2013; Crino et al., 2015). This is in part because the environmental conditions during pre- or post-natal development can "program" the HPA-axis to exhibit altered responsiveness in many vertebrates, including birds (Spencer et al., 2009; Banerjee et al., 2012; Schmidt et al.; 2014). For example, zebra finches with experimentally elevated CORT during early post-natal development (12-28 days post-hatch (DPH)) had higher stress-induced CORT levels in response to an acute stressor as adults relative to control finches (Spencer et al., 2009). Due to this ability of developmental conditions to promote variation in the adult glucocorticoid response which influences stressor tolerance and resilience, the HPA-axis likely plays a significant role in adaptive developmental plasticity in vertebrates (Vitousek et al., 2018; Schoenle et al., 2018; Schoenle et al., 2021).

While the endocrine system likely plays a large role in mediating the adaptiveness of developmental stressors, evidence indicates that such plasticity is driven by a suite of interconnected physiological and molecular changes rather than a single mechanism. For example, glucocorticoid levels are tightly linked with other aspects of the stress response such as oxidative stress. Reactive oxygen species (ROS) are produced by the mitochondria as an unavoidable byproduct of aerobic cellular metabolism under benign conditions, however, ROS levels also increase with stressor exposure and glucocorticoid elevations (Finkel and Holbrook, 2000; Costantini et al., 2008; Monaghan et al., 2009; Costantini et al., 2011). Oxidative stress describes an imbalance between reactive oxygen species (ROS) and the antioxidant defenses of an organism (Finkel and Holbrook, 2000; Costantini et al., 2011). Such an imbalance can lead to an increase in oxidative damage to endogenous molecules such as proteins, lipids, and DNA

(Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2015). The accumulation of such damage leads to the dysfunction of cells and tissues over time and is proposed as one of the main causes of ageing (Harman, 1981; Finkel and Holbrook, 2000). To curb the accumulation of oxidative damage, organisms produce endogenous antioxidant enzymes which react with and neutralize ROS (Finkel and Holbrook, 2000). The oxidative status of an organism is also influenced by other stress response mechanisms. For example, prolonged glucocorticoid secretion can inhibit antioxidant enzyme activity and cellular repair mechanisms, while upregulation of heat-shock proteins can help cope with protein dysregulation caused by oxidative stress (O'Brian et al., 1993; Liu et al., 1997; Feder and Hofmann, 1999; Flint et al., 2007; Cheng et al., 2018).

Cells exhibit a biphasic response to ROS known as mitochondrial hormesis, where moderate levels of ROS concentrations initiate signaling processes that benefit mitochondrial and cellular function (Handy and Loscalzo, 2012; Ristow and Schmeisser, 2014; Zhang et al., 2018; Hood et al., 2018; Ristow and Schmeisser, 2014). At moderate levels, ROS stimulate cell proliferation and generate protective effects through the upregulation of antioxidant enzymes, lysosomes, heat-shock proteins, and the removal of damaged organelles or cells which maintains systemic function (Grune et al., 1997; Galluzzi et al., 2012; Pickering et al., 2012). Moreso, mild levels of oxidative stress can provide adaptations to stress in *vivo*, resulting in decreased tissue damage and increased survival (Sitte et al., 2003; Ristow and Schmeisser, 2011; Pickering et al., 2013; Raynes et al., 2016; Pomatto et al., 2017). For example, human lymphocytes exposed to either a mild heat stressor or low ROS levels demonstrated an effect similar to conditioning hormesis and exhibited increased survival when later exposed to high ROS levels (Marini et al., 1996). Like the glucocorticoid response, variation in the adult antioxidant defense system and resistance to oxidative stress can be explained by an individual's developmental environment

(Atanasova et al., 2009; Costantini, 2014; Noguera et al., 2015). In birds specifically, pre- and post-natal exposure to stressful environments and elevated glucocorticoids have been demonstrated to impact oxidative balance in adulthood (Marasco et al., 2013; Homberger et al., 2013; Zimmer and Spencer, 2015). While many of the effects of developmental stressors on adult oxidative balance have been detrimental, stressor exposure of the correct timing, intensity, and duration may result in a persisting alteration in antioxidant enzyme production that is beneficial. However, it is still unclear as to what extent developing organisms could adaptively adjust their antioxidant defense phenotype in response to environmental cues in a hormetic manner, increasing tolerance to stressors.

Thermal stress

When evaluating the potential adaptiveness of developmental plasticity one key component in the experimental design is the selection of the type of environmental cue. The environmental stressor used in experimental studies should be within the range expected to be seen in nature and known to induce variation in phenotypic traits. In this way, organisms may be more likely to express plastic responses that have evolved in the species, making laboratory experiments more biologically relevant (Groothuis and Taborsky, 2015). In the research done for this dissertation we utilized elevated ambient temperatures as our environmental cue of choice. Thermal biology significantly influences the evolution of species as well as life-history at the level of the individual (Thomas et al., 2006; Chevin et al., 2010). Elevated ambient temperatures are considered an ecologically relevant stressor, as many organisms can be exposed to large temperature fluctuations under natural conditions. Organisms attempt to cope with the negative effects of heat through the initiation of the stress response, resulting in increased levels of glucocorticoids, antioxidant enzymes, and heat-shock proteins (Sapolsky et al., 2000; Cheng et

al., 2018; Kang and Shim, 2021; Woodruff et al., 2022). However, when these protective mechanisms are insufficient, the increased levels of ROS resulting from the heat stress can lead to DNA damage and inhibited repair mechanisms, protein misfolding, leading to cellular dysfunction and apoptosis (Kregel, 2002; Sedelnikova et al., 2010; Kryston et al., 2011; Kantidze et al., 2016). Thus, when heat stressors are chronic or high intensity, these cellular effects may lead to the observed negative impacts of heat stress on life-history traits such as reproduction and longevity (Lin et al., 2006; Sánchez-Hidalgo et al., 2016; Walsh et al., 2019). However, organisms with a phenotype adapted to have a higher tolerance to heat stressors could circumvent such detrimental effects on life-history traits. Such adaptive plasticity could occur through the thermal conditions experienced during development. Heat stressors have been demonstrated as strong drivers of developmental plasticity both within and across generations, generating variation in adult physiology and traits as traits such as growth, reproduction, and longevity (Le Bourg et al., 2001; Salinas and Munch, 2012; Wilsterman et al., 2015; Donelson et al., 2016; Stier et al., 2022).

Fitness-related traits and trade-offs

Another key component of experimental design when evaluating the potential adaptiveness of developmental plasticity is the selection of traits that are utilized to measure performance later in life. The response traits selected should affect fitness under natural conditions in the study species and capable of being measured under different manipulated environmental conditions (e.g., benign and stressful) to be effective proxies for fitness (Groothuis and Taborsky, 2015). Many studies measure key life history traits such as reproductive output and/or longevity due to their strong correlations with fitness and predicted trade-offs between growth, reproduction, and self-maintenance (Stearns, 1977; Sheriff and Love,

2013). However, studies examining both reproduction and longevity show mixed results with regards to trade-offs between these two traits (Le Bourg et al., 2000; Cardoso et al., 2002; Calabrese and Baldwin, 2003; Calabrese and Blain, 2005; Le Bourg, 2005; Sørensen et al., 2007).

Understanding trade-offs between traits is invaluable in the study of adaptive developmental plasticity, as while the phenotypic change may buffer the negative effects of stressors on one trait, it may come at a cost to another life-history trait that potentially constrains its evolution (Stearns, 1977; Partridge and Sibly, 1991; DeWitt et al., 1998). For example, *A. polyacanthus* reef fish exposed to elevated temperatures during development expressed reduced metabolic rate as compensation at the cost of decreased body size (Donelson et al., 2010). However, studies examining potential trade-offs of adaptive developmental plasticity often have limited variation of response traits measured, potentially missing the presence of trade-offs. Studies that do so in slower-reproducing species with longer lifespans are limited, even though they may be more likely to exhibit adaptive developmental plasticity than faster-reproducing species with shorter lifespans due to different characteristics of life-history traits (Marshall and Uller, 2007). Trade-offs between life-history traits are likely mediated by physiological mechanisms also involved in the stress response such as glucocorticoids and oxidative stress, underlying their strong links to growth, metabolism, reproduction, and senescence (Wingfield et al., 1998; Sapolsky et al., 2000; Costantini et al., 2008; Monaghan et al., 2009). As previously discussed, these are also likely involved in the underlying mechanisms of adaptive developmental plasticity, and specifically conditioning hormetic effects. As such, studies must measure both cellular and life-history traits simultaneously to avoid non-detection of effects, as

different traits and species have different capabilities for plasticity. However, studies that satisfy these criteria in endothermic vertebrates are extremely limited.

Dissertation goals and study species

In this dissertation I address research gaps in the understanding of developmental stressors and how they induce adaptive phenotypic adjustments in an avian species. I seek to examine the underlying changes in physiology regulating such potential adaptive developmental plasticity and the relationship of these parameters with ability to buffer the negative effects of a stressor on key fitness-related traits. Moreso, I seek to examine whether these changes in physiology incur trade-offs between traits under benign and/or stressful adult conditions. Finally, I address whether maternal exposure to developmental stress can also induce an adaptive phenotypic adjustment in their offspring. To do this, I exposed zebra finches (*Taeniopygia castanotis*) to a repeated mild heat stressor (38° C) during post-natal juvenile development. Then as adults, the birds were then exposed to a high heat stressor (42° C) in a 2x2 factorial design. Zebra finches (formerly *Taeniopygia guttata*) are a small altricial passerine species endemic to primarily arid areas of Australia that are believed to be at under threat of population decline as a result of an increased risk of mortality events due to heat waves (Conradie et al., 2020). Zebra finches have been widely utilized in stress and reproductive physiology research in the laboratory (Zann, 1996; Perfito, 2010). This extensive use is likely because they are easily kept and bred in captivity and quickly reach sexual maturity (Zann, 1996; Griffith et al., 2017; Griffith et al., 2021). Moreso, there are detailed descriptions of the species thermoregulatory physiology and have been utilized in both the laboratory and the field for thermal stress research (Calder, 1964; Olsen et al., 2006; Andrew et al., 2017; Cooper et al., 2019; Cooper et al., 2020; Wojciechowski et al., 2021; Ton et al., 2021)

Research questions and dissertation components

My dissertation is composed of five data chapters that examine variation in different fitness-related traits following a high intensity heat stressor as adults, and the relationship between these traits and the underlying physiology influenced by an experimental mild heat conditioning during development.

For my dissertation, I aim to look at the following questions using the zebra finch (*Taeniopygia castanotis*) as a study organism.

1) Can exposure to an experimental mild heat conditioning during development induce an adaptive phenotypic adjustment, preventing or attenuating the negative impacts of a high intensity thermal stressor on life-history traits such as immune function, reproduction, longevity, and cellular damage?
2) What underlying physiological changes such as altered glucocorticoids, antioxidant enzymes, or heat-shock protein expression act as mechanisms mediating such adaptive developmental plasticity/hormetic effects?
3) What are the relationships between these traits? Is there an underlying cost to the plasticity, and to what extent do the future environmental conditions influence the presence of costs and trade-offs (e.g. environmental matching)?
4) When females are exposed to the mild heat conditioning as juveniles, does it induce a maternal effect, influencing the phenotype of their offspring? If so, will these maternal effects adaptively prepare offspring to cope with elevated ambient temperature pre-natally?

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Chapter 2: Early stress priming and the effects on fitness-related traits following an adult stress exposure

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Summary statement:

Zebra finches exposed to a mild heat stressor during development had decreased wound healing ability and increased clutch viability in adulthood, indicating a trade-off between immune and reproductive function due to a cost of hormetic priming when there is an environmental mismatch.

Abstract

Developmental stressors can have strong effects that persist well into adulthood, and are generally seen as detrimental. However, recent work suggests that a mild developmental stressor can have beneficial effects by preparing the organism to better withstand negative impacts when exposed to high levels of the stressor later in life, also known as a conditioning hormesis. Still, little is known about the influence of such hormetic effects on fitness-related measures. We hypothesized that exposure to a mild stressor during development will protect individuals later in life from the negative effects of a high heat stressor on immune function and reproduction. To test this hypothesis, we subjected zebra finches (*Taeniopygia castanotis*). to a repeated mild heat stressor (38° C) as juveniles for 28 days. As adults, the birds were then exposed to a high heat stressor (42° C) for 3 consecutive days and we examined the effects on immune function via wound healing, and on female reproductive output. We found that females exposed to the mild heat conditioning as juveniles had slower healing wounds as adults when they were not exposed to the high heat treatment. When looking at the time it took for females to lay their first egg, we saw a pattern indicative of conditioning hormesis. However, with regards to clutch viability, we saw a stimulatory effect of thermal treatments. Together, our findings indicate potential adaptive effects of mild developmental stressors, but also potential costs when there is a mismatch between the early and adult environments.

Introduction

The developmental period of an organism is sensitive to changes in the surrounding environment, which can have strong and persistent phenotypic effects (Mousseau and Fox, 1998). Numerous studies have examined the long-lasting detrimental effects of poor early environments, such as through manipulation of food availability, exogenous glucocorticoid administration, and increased brood size (Francis et al., 1999; Spencer et al., 2009; Wada et al., 2015). In birds, such developmental stressors have been shown to decrease growth, immune function, and learning ability, reduce longevity, and dampen sexual selected traits in adulthood (Spencer et al., 2005; Harris and Seckl, 2011; Monaghan et al., 2012; Chin et al., 2013). However, developmental stressors may not always be maladaptive, and that the effect depends on both the magnitude of the stressor and the adult environment of an organism. Developmental stressors have the potential to increase fitness by adjusting an organisms' phenotype to prepare them for the conditions they will experience in adulthood (Monaghan, 2008). For instance, an early stressor can calibrate the stress response system, such as through hypothalamic-pituitary-adrenal axis programming, altering the threshold for triggering a stress response and the magnitude of future responses (Romero et al., 2009). These are also known as predictive adaptive responses, and for these adaptive effects to present themselves, it is necessary for the environment to be relatively stable during an organisms' lifetime (Gluckman et al., 2005). That is, if the adult environment does not match the early environment which caused the phenotypic adjustment, then this change will be detrimental rather than adaptive (Environmental matching hypothesis - Monaghan, 2008; Harris and Seckl, 2011) (Fig. 1A).

The adaptiveness of developmental stressors can also be explained by a dose-dependent effect, through a phenomenon known as hormesis (Mattson and Calabrese, 2010). The classical

definition of hormesis originated from toxicology and health research, and describes a biphasic, or inverted U-shaped curve, where the response of an organism changes with the level (i.e., dose) of the toxin or stressor (Mattson and Calabrese, 2010). Essentially, mild levels have a beneficial effect, whereas high levels are detrimental (Costantini et al., 2010; Mattson and Calabrese, 2010). More recently, the term hormesis has been given a second meaning. In this second use of the word, hormesis occurs when exposure to a mild stressor primes an organism to cope with a higher severity of the stressor later in life (Fig. 1B). This form of adaptive phenotypic plasticity has been referred to as conditioning hormesis, and essentially gives an organism a persistent increase in tolerance to a stressor (Calabrese et al., 2007). This conditioning hormesis can be induced through mild exposure at any point in life, though there is some evidence that the effect is stronger when exposure occurs during the more sensitive period of development (Bartling et al., 2003; Honma et al., 2003; Le Bourg et al., 2004). Therefore, exposure to a mild stressor early in life may result in adaptive developmental plasticity, and specifically through the induction of a conditioning hormetic effect.

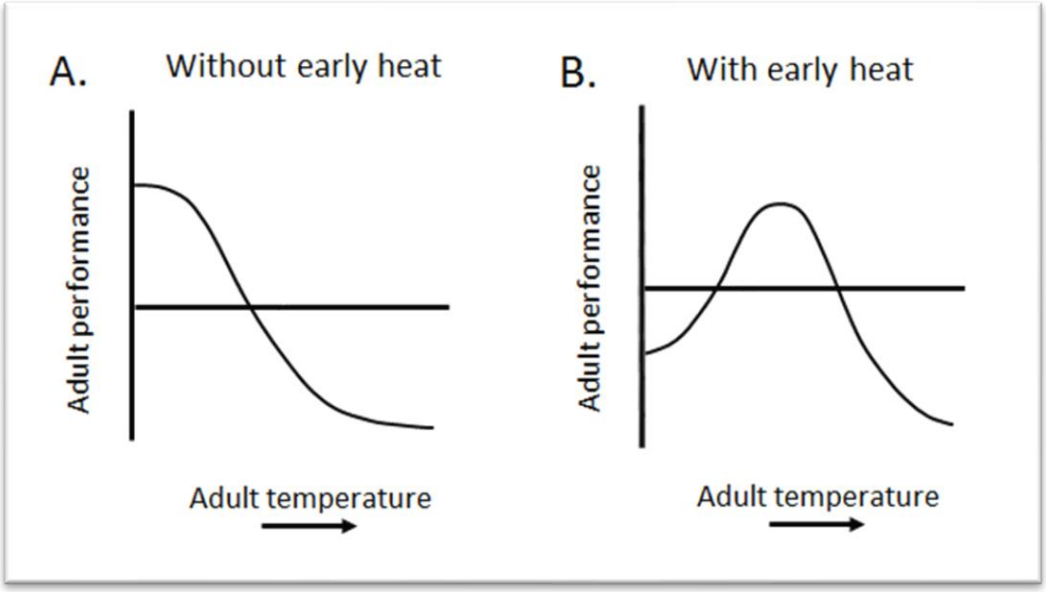

Fig. 1. Conceptual models of the relationship between adult performance and temperature in context of early life environment. When the curve is above the center line, it represents a stimulatory effect on adult performance, whereas when the curve falls below the line, it represents an inhibitory effect. Panel A represents the expected decrease of performance with increasing temperature when the animal was not "primed" by heat exposure early in life. Panel B represents the expected adult performance/temperature relationship in a conditioning hormesis context. In this model, early heat exposure "primes" the animal to have increased performance at an otherwise detrimental temperature. Modified from Carter et al. (2013) and Monaghan (2008).

Ambient temperatures are known to have phenotypic effects on a variety of taxa, and indeed, studies have demonstrated thermally-induced hormesis (Mattson and Calabrese, 2010). Heat stress is considered an ecologically relevant stressor, as animals in the wild can be exposed to large temperature fluctuations with the potential of inducing a stress response and impacting the phenotype of an individual. One example of thermally-induced conditioning hormesis is a study done in which zebra finches (*Taeniopygia castanotis*) exposed to a mild heat stressor of 38° C as juveniles suffered less oxidative damage from exposure to a higher heat stress in adulthood, compared to juvenile controls (Costantini et al., 2012). However, this priming effect can come with a cost when there is an environmental mismatch later in life, as it was later found that the heat conditioned birds that did not receive the high heat stress as adults had lower longevity (Costantini et al., 2014). This supports the idea that all fitness-related traits cannot be

increased simultaneously through hormesis, and that resource trade-offs may occur between traits such as survival and reproduction.

Using zebra finches, we tested the hypothesis that prolonged exposure to a mild heat stressor as juveniles has a conditioning hormetic effect, preventing negative effects on fitness-related traits when exposed to a high-heat stressor as adults. In birds, heat stress can suppress immune function (Mashaly et al., 2004; Quinteiro-Filho et al., 2010). One integrative and ecologically relevant method of measuring immune function is wound healing ability (Martin, 1997; French et al., 2006), which can be affected by thermal challenges in birds (Carter et al., 2013). In this study, we used wound healing as a measure of overall innate immunity to quantify the ability of finches to cope with a heat stressor. We predicted that birds that received mild heat conditioning as juveniles would have faster healing wounds when exposed to a high heat stressor as adults, compared to those that did not receive the mild heat.

Reproduction is energetically costly, and when an organism is faced with an increase in energetic demands, trade-offs are made between self-maintenance and current reproduction (Stearns, 1992). Exposure to a stressor can cause such a predicament, and it has been shown that glucocorticoids can mediate this trade-off by suppressing reproductive physiology and behavior (Ching, 1983; Suter and Schwartz, 1985; Carter, 1992ab; Negro-Vilar, 1993). In birds, oxidative stress and/or glucocorticoids are shown to suppress reproductive performance, such as by decreased egg mass and hatching success. Thermal stress more specifically can result in a delayed onset and rate of laying, decreased clutch size, and decreased fertility (Keirs, 1982; Mcdaniel et al., 1995; Salvante et al., 2007; Bize et al., 2008). In this study we used female reproductive output to evaluate a finch's resilience to a high heat stressor. We predicted that heat conditioned females would have shorter onset of laying, and higher average clutch size, egg

mass, and clutch viability after exposure to a high-heat stressor than those that were not conditioned as juveniles. Relatively little is known about which fitness-related traits are subject to conditioning hormetic responses. This study provides a more holistic view of the extent hormetic effects have in preventing detrimental impacts on fitness-related traits when confronted with a stressor.

Methods and materials

Diet and husbandry

Experimental birds were obtained by breeding captive zebra finches (*Taeniopygia castanotis*) held in our aviary at Auburn University. The 32 pairs were held in tower cages (38.10 cm Width x 45.72 cm Depth x 45.72 cm Height) and received food and water *ad libitum*, with a diet composed of mixed seeds (Kaytee Supreme (finch), Chidton, WI), grit, and cuttlefish bone. Each individual cage was fitted with a nest box (19.5x14.5x14.5 cm) bedded with shredded paper covered with irradiated hay, and pairs were sprayed with water twice per day to promote the onset of breeding behavior. They were also given chopped spinach and a vitamin supplement (Wild Harvest, Blacksburg, VA, USA) in water once per week. To supplement their diet, pairs received one tablespoon of egg food mixture per day up until the oldest nestling was 5 days post-hatch (DPH), at which point they were given two tablespoons per day until the youngest nestling reached nutritional independence at 35 DPH (Wada et al., 2015). Upon hatching, birds were marked to allow individual identification by cutting the down feathers in recognizable patterns. At 10 DPH, nestlings were weighed and given unique ID bands. Offspring were separated from their parents approximately three days before the start of the juvenile stage treatment. All animal husbandry and experimental protocols were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016-2826).

Juvenile mild heat conditioning

A total of 88 offspring (48 females and 40 males) underwent the juvenile conditioning treatment. Sex was determined by plumage and treatment groups were semi-randomly assigned within nest, hatch order, and sex, to either a mild heat or control group. Following a conditioning protocol previously established (Costantini et al., 2012), half of the juveniles at 42-45 DPH (Fig. 2) underwent a mild heat treatment by being exposed to a temperature of 38° C for 3 hours per day in a brooder (TLC-50, Brinsea Products Inc., FL), every other day for a total of 14 times. The temperature of 38° C was chosen as a mild heat stressor because it is near the upper critical temperature, but still within the thermoneutral zone for the species (Calder, 1964). Although within the thermoneutral zone, at ambient temperatures higher than 30°C, body (cloacal) temperatures begin to rise toward mild hyperthermia allowing the species to dissipate heat in order to limit water loss (Calder, 1964). This increase in body temperature may be accompanied by initiation of stress responses such as heat shock protein synthesis and glucocorticoid secretion (Wingfield et al., 1998; Feder and Hoffmann, 1999) without resulting in oxidative damage or a decrease in growth (Costantini et al., 2012). On treatment days, groups of 3-7 finches were placed in brooders starting between 8:30-9:30 a.m., where they received *ad libitum* cold water which was replaced after the first and second hour of the three-hour treatment period (Costantini et al., 2012; Finger et al., 2017). The remaining juveniles served as a control group and were placed in brooders set to room temperature (21-23° C) in the same fashion as the mild heat group. At the end of the treatment period, birds were placed in single sex cages (1-3 birds per cage) and provided the same diet as previously described. The birds remained there until they reached 170-180 DPH at which point the adult stage treatment began.

Adult high heat stress

In a 2x2 factorial design, half of the birds from both the juvenile mild heat and control groups underwent a high heat stress treatment of 42° C for 3 hours/day for 3 consecutive days as described above. The temperature of 42° C was chosen as it is higher than the upper critical temperature of the thermoneutral zone for the species but still below the lethal temperature, and this temperature and duration of heat stress is shown to trigger oxidative damage in zebra finches (Calder, 1964; Costantini et al., 2012). The other half of the juvenile-stage control and mild heat groups were placed in a brooder set to the room temperature (21-23° C) in the same fashion as above to serve as an adult-stage control group. This resulted in four different treatment groups; Control juvenile – Control adult (cont:cont, 6 males and 12 females), Control juvenile – High heat adult (cont:high, 10 males and 13 females), Mild heat juvenile – Control adult (mild:cont, 13 males and 11 females), and Mild heat juvenile – High heat adult (mild:high, 10 males and 8 females). The birds were weighed both the day before the treatment began and on the last day of the heat stress. Following the last treatment bout on the third day, male birds were sacrificed for a subsequent study via isoflurane inhalation per approved protocol.

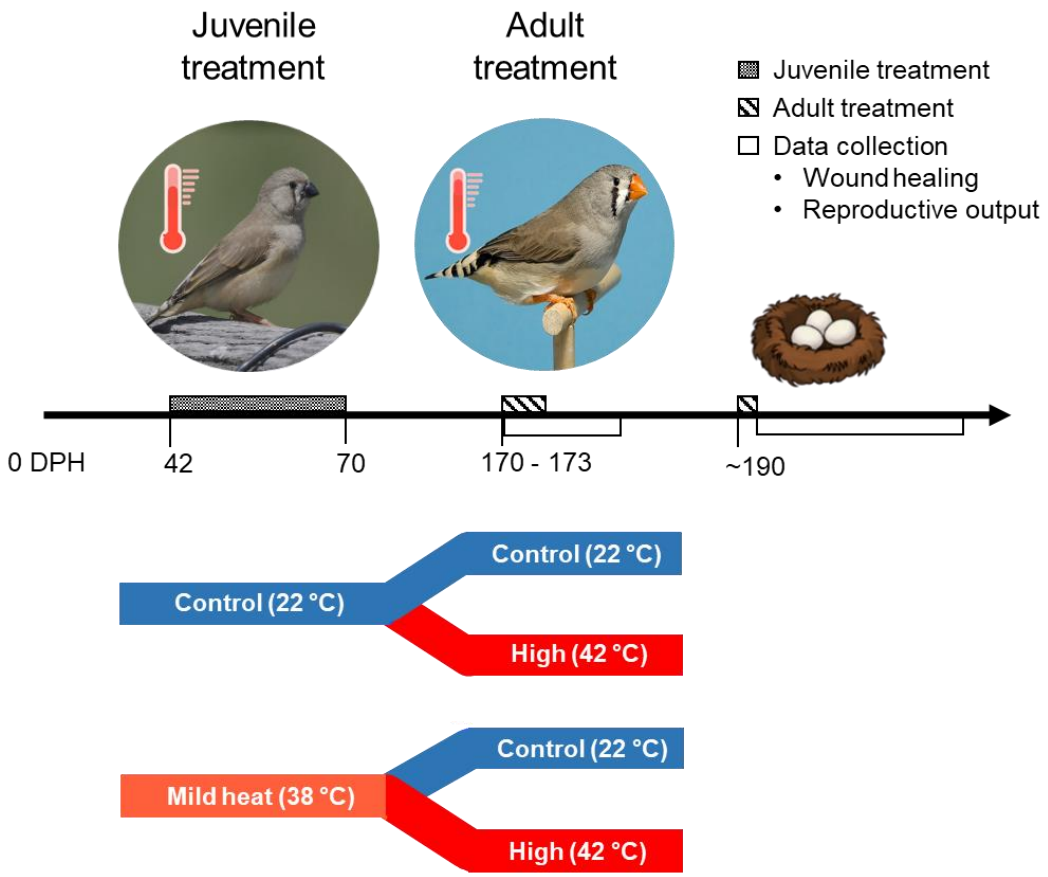

Fig. 2. Schematic timeline of treatments and collection points. Beginning at 42-45 days post-hatch (DPH), male and female finches were exposed to either a mild heat (38 °C) or control (22 °C) treatment over a 28-day period. In adulthood, starting at 170-173 DPH, these females were then exposed to either a high heat stressor (42 °C) or control temperature (22 °C) over a 3-day period in a 2x2 factorial design. Wound healing was measured during the 3-day adult treatment period and after until the wounds were fully healed. Female reproductive output was measured several weeks following the end of the adult treatment period.

Immune function

We used wound healing ability to evaluate immune function, as it has been proposed to be an ecologically relevant measurement (Martin, 1997; French et al., 2006). To do this, a skin excision approximately 3.0 mm in diameter was made by pinching and lifting a portion of the skin on each thigh using forceps and cutting it with fine scissors. Prior to the excision, the skin was cleaned with 70% ethanol, and a small amount of a topical lidocaine gel was applied as an anesthetic. Wounds were made the day before the adult thermal trials began, and wounds were

photographed with a ruler as a scale every day until the wounds were fully healed (44 females), or until the day the adult treatment ended (39 males). Area (mm^2) was quantified by measuring the perimeter of the wound using ImageJ v1.51j (National Institutes of Health, USA) and a wound was considered fully healed when no scab or open tissue was visible and all skin was reconnected. Wounds that were made too large or small, or had scabs torn off during the healing process were not included in the final analysis.

Female reproductive measures

To quantify female reproductive effort, four parameters of reproductive output were measured: the time elapsed for a female to lay her first egg (in days), egg mass (g), brood mass (g), clutch size, and clutch viability. Females were paired with non-experimental males approximately 3 weeks after the end of the adult treatment, and on the day of pairing females were exposed to a single bout of the adult treatment (control or high heat) as described above. Following the same breeding protocol as described above, nest boxes were checked once per day to determine the date on which the nest was completed by the male. The count for days to first egg began once nest construction by the male was completed, rather than when the male and female were initially paired. Any males that did not initiate nest construction within 3 weeks were removed and replaced with another non-experimental male. All eggs were collected, weighed, and placed in an incubator the day they were laid so viability could be quantified regardless of parents' incubation behavior. The first egg of each nest was removed and replaced in the nest with a false egg made of forming clay. Any subsequent eggs laid and removed were not replaced.

Statistical Analyses

Data analysis was done using RStudio (R Core Team, 2015) and statistical significance was recognized as $p \leq 0.05$ for all models. All statistical models included juvenile and adult treatment as fixed effects, genetic nest as a random effect to account for parental effects, and a juvenile by adult treatment interaction term. Starting with the full model, a stepwise process was used to remove independent variables with $p > 0.1$. The juvenile*adult interaction was kept in the models regardless of significance due to the conditional nature of our hypothesis, as inclusion of the juvenile*adult treatment interaction term cannot be decided solely on the significance of its coefficient (see Brambor et al., 2006). Wounds made on the left and right legs were analyzed separately, as initial wound sizes were on average significantly larger on the right, and rates of healing were different between the two. Three models were used to evaluate wound healing, with each including initial wound size as a covariate. A linear mixed-effects model was used to analyze the rate of healing (mm^2/day) during the three days of adult treatment period (i.e. treatment healing rate), with sex as an added fixed effect. The second wound healing model was also a linear mixed-effects model, and was used to evaluate the rate of healing (mm^2/day) from the end of the three-day adult treatment until the wound was fully healed (i.e. post-treatment healing rate). A third model was used to evaluate the amount of time (in days) it took for the wound to fully heal (i.e. overall healing time), for which a Cox mixed-effects model was used. Sex was not included as a fixed effect in the latter two models, as only female data are available for this time period.

The time to first egg was analyzed using a Cox mixed-effects model. Analysis of clutch size was done using a generalized linear mixed-effects regression with Poisson distribution, and egg mass, brood mass, and clutch viability were analyzed using linear mixed-effects models.

Results

Wound healing

During the adult treatment period (Day 0-3), wounds healed at an average rate of 4.2 $x10^{-3}$ ($\pm$5.4$x10^{-4}$) mm^2/day. Healing rates during this period were not significantly affected by juvenile or adult treatment on either leg, nor was there any significant interaction between them (Left – juvenile: t = -3.8, P = 0.705; adult: t = -0.74, P = 0.942; J*A: t = 0.42, P = 0.675. Right – juvenile: t = -0.24, P = 0.809; adult: t = 0.61, P = 0.544; J*A: t = 0.81, P = 0.421). Sex also had no effect on healing rate of either leg (Left: t = -0.76, P = 0.453; Right: t = -1.00, P = 0.320). The wounds of some individuals had an initial increase in area before they began to reduce in size. We quantified the degree of this inflammation by dividing the maximum size of the wound by its initial size. On the right leg, which on average had a larger initial wound size, males had higher inflammation than females (t = 2.8, P = 0.009), though there was no effect of treatment or interaction (juvenile: t = 0.74, P = 0.461; adult: t = 0.056, P = 0.956; J*A: t = 0.003, P = 0.998).

After the adult treatment had ended (Day 3 to fully healed, post-treatment rate) we again measured the rate of healing in female finches, which was on average 1.3 $x10^{-2}$ ($\pm$4.5 $x10^{-4}$) mm^2/day during this period. On the left leg, there was a marginal conditional main effect of juvenile treatment, and a marginal juvenile*adult treatment interaction (LMM: juvenile: t = -1.82, P = 0.0890, adult: t = 0.800, P = 0.4368; J*A: t =1.81, P = 0.0911). Subsequent pairwise comparisons showed that among the females exposed to the mild heat conditioning as juveniles (mild:cont, mild:high), the wound on their left legs healed at a rate of 2.91 $x10^{-3}$ mm^2/day ([9.45 $x10^{-4}$, 4.87 $x10^{-3}$], $\pm$95% CI) slower if they were exposed to the control treatment (mild:cont) rather than the high heat treatment as adults (mild:high) (Fig. 3) (LMM: t = 3.00, d.f. =15, P = 0.0090). Surprisingly, the wound on the left leg of these mild:cont females also healed at a rate 2.21 $x10^{-3}$ mm^2/day ([4.53 $x10^{-4}$, 3.96 $x10^{-3}$], $\pm$95% CI) slower than those on non-conditioned

females that were exposed to the high heat treatment as adults (cont:high) (LMM: t = 1.82, d.f. =15, P = 0.0224). However, no such patterns were observed on the right leg (LMM: juvenile: t = 1.1, P = 0.288; adult: t = -0.06, P = 0.951; J*A: t = 0.54, P = 0.600).

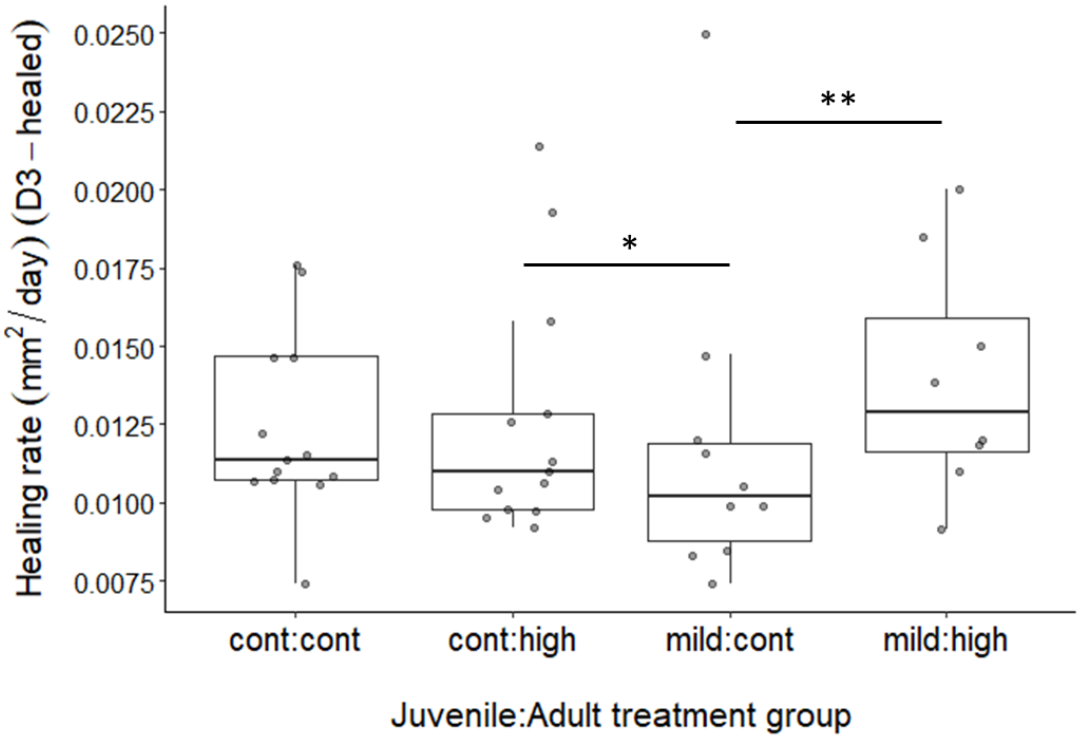

Fig. 3. The rate of healing (mm^2/day) on the left leg from the end of the 3-day adult treatment until the wound was fully healed. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control - 22 °C or mild heat - 38 °C) and the second word denotes which treatment they were exposed to as adults (control - 22 ° or high heat - 42 °C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

We analyzed the overall time (in days) it took for the wound to completely heal, which took on average 9.1 (±0.14) days. We saw that among the females exposed to the mild heat conditioning as juveniles (mild:cont, mild:high), the wound on their left legs healed 32.7% ([1.13, 9.44], ±95% CI) slower if they were exposed to the control treatment (mild:cont) rather than the high heat treatment as adults (mild:high) (COXME: β = 1.18, ±95% *CI* = [0.123, 2.25],

z = 2.19, *P* = 0.029) (Fig. 4). The wound on the left leg of these mild:cont females also healed 29.8% ([1.17, 7.60], ±95% CI) slower than those on non-conditioned females that were exposed to the high heat treatment as adults (cont:high) (COXME: β = 1.09, ±95% *CI* = [0.153, 2.03], z = 2.28, *P* = 0.023). On the right leg however, no such trend was observed (COXME: juvenile: β = 0.674, ±95% *CI* = [-0.226, 1.57], z = 1.47, *P* = 0.1400; adult: β = 0.442, ±95% *CI* = [-0.390, 1.27], z = 1.04, *P* = 0.3000; J*A: β = -0.359, ±95% *CI* = [-1.60, 0.886], z = -0.57, *P* = 0.5700).

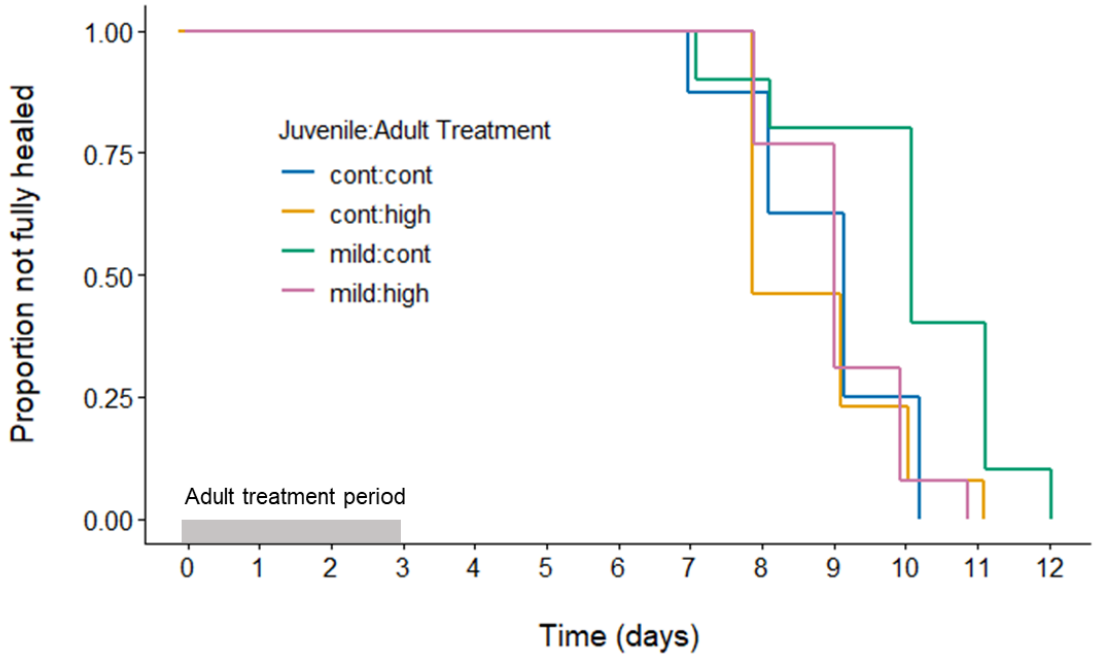

Fig. 4. The amount of time (in days) for wounds on the left leg to fully heal. The graph shows the proportion of all females of a group being completely healed on a given day. The x-axis denotes the time (in days) since the initial wound was made (170 DPH). In the legend, the first word denotes the treatment the mothers received as juveniles (control - 22 °C or mild heat - 38 °C) and the second word denotes which treatment they received as adults (control - 22 ° or high heat - 42 °C).

Female reproductive output

Out of the 40 experimental females paired to breed, 38 successfully laid fertile eggs (1 cont:cont and 1 cont:high female did not successfully lay). When examining the time (in days) it took for females to lay their first egg once the nest was made, we saw a significant effect of our experimental treatments. We saw that among the females exposed to the high heat treatment as

adults (cont:high, mild:high), females exposed to the control treatment as juveniles (cont:high) took 33.4% ([1.13, 9.82], ±95% CI) longer to lay their first egg than females exposed to the high heat treatment as adults (mild:high) (COXME: β = 1.20, ±95% *CI* = [0.125, 2.28], z = 2.19, *P* = 0.029). Furthermore, these non-conditioned females exposed to the high heat treatment as adults (cont:high) took 39.7% ([1.40, 11.2], ±95% CI) longer to lay their first egg than females exposed to the control treatment as both juveniles and adults (cont:cont) (COXME: β = 1.38, ±95% *CI* = [0.340, 2.42], z = 2.60, *P* = 0.0093).

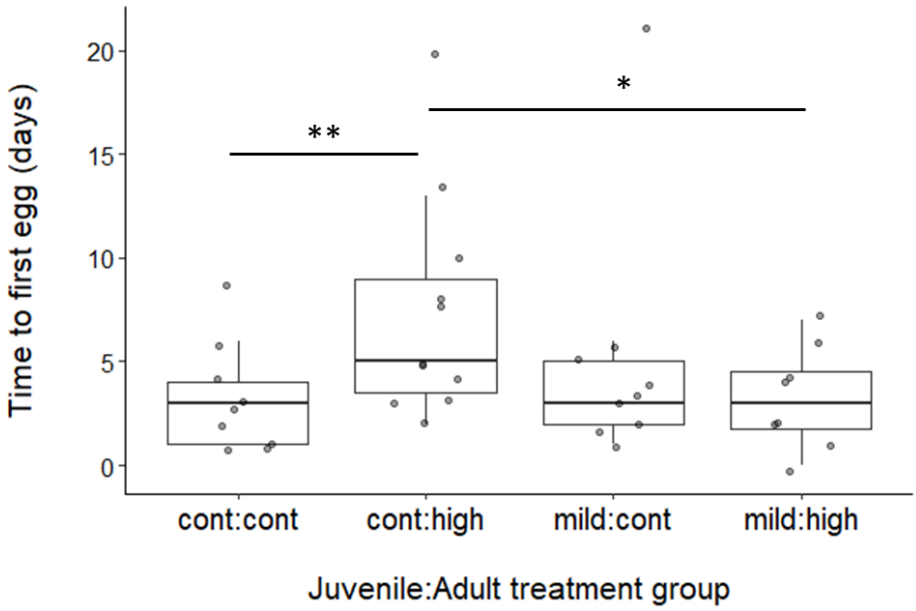

Fig. 5. The time (days) it took for experimental females to lay their first egg once the nest was made. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control - 22 °C or mild heat - 38 °C) and the second word denotes which treatment they were exposed to as adults (control - 22 ° or high heat - 42 °C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Clutch size showed no effect of treatments or their interaction (GLM: juvenile: *z* = 0.53, *P* = 0.596; adult: *z* = -0.99, *P* = 0.324; J*A: *z* = -0.51, *P* = 0.608) with females on average laying 4.83 ±0.44 eggs in their clutch. For egg parameters, there was no significant effect of the treatments the female received on either egg mass (LMM: juvenile: *t* = -0.20, *P* = 0.848; adult: *t*

= -0.14, *P* = 0.971; J*A: *t* = -0.143, *P* = 0.888) or brood mass (LMM: juvenile: *t* = 0.19, *P* = 0.855; adult: *t* = -0.76, *P* = 0.461; J*A: *t* = -0.54, *P* = 0.595). However, there were significant unconditional main effects of juvenile and adult treatment on clutch viability. Females that received the mild heat treatment as juveniles had a 20.3% ([±0.154], ±95% CI) more viable eggs in their clutch than juvenile controls, without accounting for the treatment they were exposed to as adults (LMM: *t* = 2.82, d.f. =14, *P* = 0.0136) (Fig. 6A). However, there was no significant unconditional main effect of adult treatment (LMM: β = 0.119, *t* = 1.64, d.f. =14, *P* = 0.1237). The interaction between juvenile and adult treatment had a marginal effect (LMM: β = -0.317, *t* = -2.11, d.f. =13, *P* = 0.0550), and pairwise comparisons showed that the cont:cont females had lower clutch viability than all other groups (Fig. 6B) (LMM: cont:cont – cont:high (β = 0.258, *t* = 2.52 , d.f. = 13, *P* = 0.0255), cont:cont – mild:cont (β = 0.364, *t* = 3.38 , d.f. = 13 *P* = 0.0049), cont:cont – mild:high (β = 0.305, *t* = 2.83 , d.f. = 13, *P* = 0.0141).

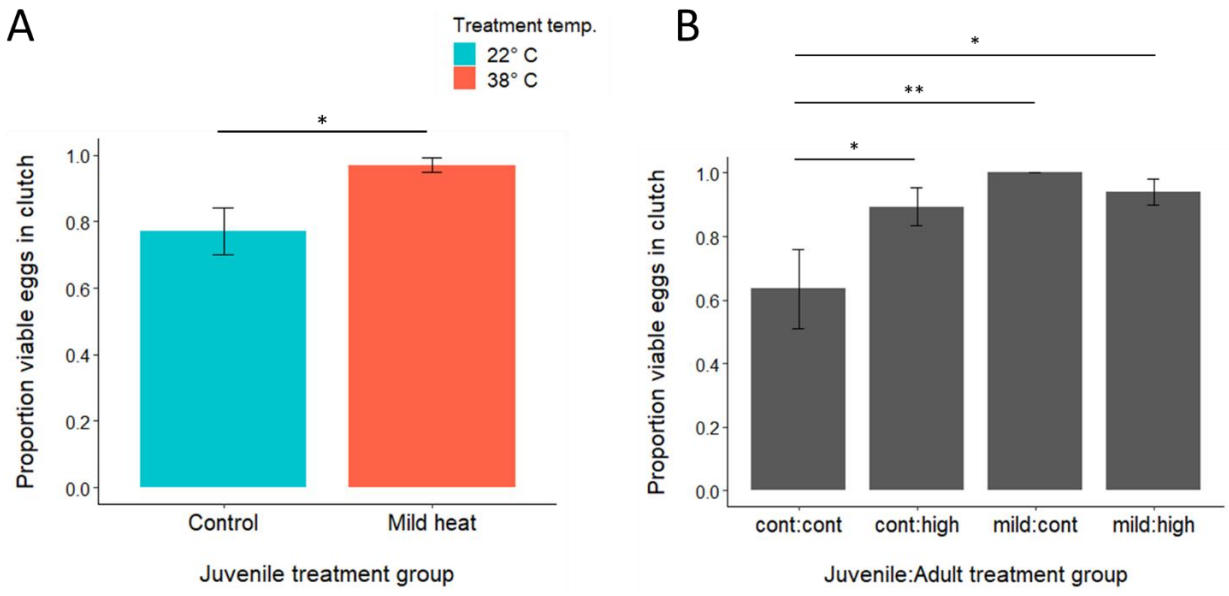

Fig. 6. The effects of treatments on the proportion of viable eggs in an experimental females clutch. (A) The main unconditional effects of juvenile treatment on the proportion of viable eggs. **(B)** Comparisons between the four discrete treatments groups representing the combinations of juvenile and adult treatment groups. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control - 22 °C or mild heat - 38 °C) and the second word denotes which treatment they were exposed to as adults (control - 22 ° or high heat - 42 °C). Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Discussion

The goal of this study was to determine if immune function and reproductive output exhibit heat-induced conditioning hormetic responses. To determine this, we treated zebra finches with a prolonged mild heat stressor as juveniles and examined the effects on wound healing and reproductive output when exposed to a high heat stressor as adults. To our surprise, conditioned females had slower healing wounds when they were exposed to the control treatment rather than the high heat stressor. When examining the reproductive output of females, some aspects demonstrated a pattern consistent with conditioning hormetic effects while others did not. Our results indicate that a mild heat stressor during development can have effects persisting into adulthood, leading to potentially beneficial changes in fitness-related traits. However, these phenotypic changes may come at a cost depending on the later life environment.

Effects of thermal treatments on wound healing

In our study, the adult high heat treatment did not have a suppressive effect on wound healing rates during the treatment period. One possibility for why did not see the predicted negative effect on wound healing ability in non-conditioned (cont:high) finches, is food availability. Except for when in the brooder, birds had *ad lib* access to seeds. Therefore, the finches may have increased food intake in order to compensate for an increase in energetic demands due to the high-heat stressor. In this way, finches could have minimized resource trade-offs caused by the high heat, allowing them to maintain immune function. Another potential explanation is based on the importance of intensity and duration in determining the effect magnitude of a stressor. The temperatures and durations of juvenile and adult treatments used were chosen because they have been previously shown to induce conditioning hormetic effects in zebra finches (Costantini et al., 2012). However, the traits they measured to determine the

impacts of the thermal stressors were parameters of oxidative stress at the tissue level, with the effects on plasma oxidative damage indicating hormetic priming. Exposure to stressors generally first elicits responses at lower levels of biological organization, and negative impacts of stress may be more readily observed at the cellular or tissue level (Romero et al., 2009). Moreso, such detrimental effects may be transient and may never lead to detrimental effects on traits at the organismal level (Wada and Heidinger, 2019). As such, while the high heat treatment may be sufficient to induce oxidative damage, it may require a longer or higher intensity heat stressor to impair some traits at a higher level of biological organization such as wound healing ability.

Unexpectedly, we saw that the females exposed to the mild heat conditioning as juveniles and the control treatment as adults (mild:cont) had slower wound healing rates than both groups exposed to the high heat treatment (mild:high and cont:high) after the end of the adult treatment period. This result generally supports one of the core tenants of predictive adaptive responses and environmental matching, namely that if the adult environment does not match the early environment which induced the phenotypic adjustment, then this change will be maladaptive (Gluckman et al., 2005; Monaghan, 2008). The slower wound healing of mild:cont females was also expressed when examining the total time (days) it took for wounds to completely heal, as again we saw that the wounds on mild:cont females took longer to heal than mild:high and cont:high females. Interestingly, we did not see the same trend in the other non-matching group (cont:high). Once again, it's possible that these cont:high females were able to buffer the negative effects of the high heat treatment on wound healing through increased food consumption and avoidance of resource allocation. However, such a strategy may not be feasible to offset costs of developmental stressors in a mismatched environment. The costs could be mediated by persisting phenotypic alterations elicited by a change in developmental trajectory,

rather than resource allocation (Natelson et al., 1988; Spencer and MacDougall-Shackleton, 2011)

Effect of heat exposure on reproduction

In our study, we predicted that without exposure to the early life mild heat conditioning, females exposed to the high heat treatment as adults (cont:high) would have the lowest reproductive output. However, we saw that many of the female reproductive measures were unaffected by juvenile or adult treatment. This may be due to the life-history strategies of the study species. Some studies have shown that zebra finches will maintain reproductive function at a cost to other physiological processes when faced with a stressor. For instance, in a study where reproductive cost was experimentally increased through manipulation of brood size, female zebra finches increased energy allocation toward reproductive effort at the cost of decreased antioxidant defense (Alonso-Alvarez et al, 2004; Wiersma et al., 2004).

One female reproductive trait that was affected in the manner we predicted was the time it took for experimental females to lay their first egg once the nest was made (latency to lay). With regards to this trait, we saw that it expressed the predicted pattern indicative of a conditioning hormetic effect. Among the females exposed to the high heat treatment as adults, those that were previously exposed to the mild heat conditioning as juveniles (mild:high) had a shorter latency to lay their first egg than those exposed to the control treatment as juveniles (cont:high). Furthermore, these non-conditioned females exposed to the high heat treatment as adults (cont:high) had a longer latency to lay than females exposed to the control treatment at both timepoints (cont:cont). This result potentially indicates that exposure to the mild heat conditioning resulted in predictive adaptive response, making finches more suited maintain reproductive performance when exposed to the high heat treatment through conditioning

hormesis. This detrimental effect of the high heat treatment on latency to lay is likely mediated by oxidative stress, which has been shown to increase as a result of this specific protocol (Costantini et al., 2012). Moreso, in birds it has been demonstrated that an experimental increase in oxidative stress prior to a reproductive bout can result in higher lay latency and decreased clutch sizes (Costantini et al., 2016). In the wild, zebra finches are opportunistic breeders that live in semi-arid regions of Australia (Zann, 1996). It's possible that longer latency to lay could mean less resources that were present at the onset of breeding (e.g. water, immature grass seeds) could be available to parents or offspring (Zann, 1996). The ecological relevance of latency to lay in zebra finches is not known, however, it has the potential to have significant effects on fitness.

Another reproductive parameter that was affected by the heat treatments was clutch viability. We saw that regardless of the time point of exposure or stressor magnitude, females exposed to increased temperatures as juveniles and/or adults (cont:high, mild:cont, mild:high) had a higher percentage of viable eggs in their clutch compared to those never exposed to elevated temperatures (cont:cont). These heat-mediated increases in clutch viability are somewhat surprising, as there is some evidence that heat stressors can decrease fertility in birds when environmental matching is not occurring (Keirs, 1982; Mcdaniel et al., 1995). It is interesting that even exposure to the high heat treatment alone resulted in higher clutch viability, which points to a potential hormetic effect of heat in the classic stimulatory toxicological meaning.

One possible explanation for these effects of heat stress on clutch viability is that the exposure caused a shift in life-history strategy in female finches with "mismatched" juvenile and adult treatments. Other studies have demonstrated that the high heat treatment causes oxidative

damage as well as a potential cost resulting from exposure to the mild heat conditioning (Costantini et al., 2012; Costantini et al., 2014). It has been shown that zebra finches with mismatched juvenile and adult treatments (i.e., cont:high, mild:cont) had reduced survival compared to those that had matching treatments (i.e., cont:cont, mild:high) (Costantini et al., 2014). This decrease in longevity may trigger a change in the finches "pace-of-life" and the strategy in which they allocate resources. A shorter life means potentially a lower number of reproductive opportunities, increasing the value of each breeding bout. This would cause parents to allocate more resources to reproduction rather than current self-maintenance (Stearns, 1992). Such strategies have been previously documented in birds, where species with lower survival and smaller clutches are more likely to mitigate the risk on their offspring at a cost on self-maintenance when faced with a challenge (Ghalambor and Martin, 2001). However, this positive effect of the high heat treatment on clutch viability and its detrimental effect on lay latency are somewhat contradictory, making interpretations difficult.

Conclusions

The developing environment can have persistent, or even instructive effects on an organisms' phenotype which can be adaptive depending on the later life environment. Hormesis shows the potential for stressors to act beneficially in a dose-dependent manner, allowing animals to maintain fitness when faced with a harsh environment. In this study, we investigated whether a mild heat stressor in early life can induce conditioning hormesis, mitigating the negative effects of a high heat stressor on immune function and reproduction in adulthood. Our results did not show the expected pattern of conditioning hormetic responses for immune function, but rather a potential cost of hormetic priming when there is a mismatch between phenotype and the adult environment. With regards to aspects of female reproduction, we saw

conflicting patterns between traits. While the effects of treatments on latency to lay suggest a conditioning hormetic effect and a negative impact of the high heat treatment, the effects on clutch viability suggest a potentially stimulatory role of heat exposure. More studies are needed to determine whether the increase in clutch viability was due to a shift in life-history strategy, an increase in stressor tolerance, or a potential cross-stressor hormetic effect. Our results suggest a possible trade-off between oxidative stress and reproductive output, mediated by a cost of hormetic priming when the early and later life environment do not match. Further work is needed in order to answer questions regarding potentially adaptive developmental stressors, such as how the magnitude of effect can change across life-history and the traits they influence. Studies evaluating multiple traits at different biological levels may be key, as traits have different capabilities for plasticity and timepoints at which costs and benefits are observable. Such studies along with investigations of potential mechanisms behind adaptive effects, and whether they can extend across different stressor types, will present a clearer picture of the adaptive potential of stressors.

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

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Data accessibility statement

All data used for analyses are available in the main text or supplementary materials and will be available in a digital data repository by manuscript publication.

Conflicting Interests

The authors declare no conflicting interests.

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Chapter 3: Developmental thermal conditioning regulates oxidative state and beak coloration in response to thermal stressors in adulthood

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Summary statement: In captive zebra finches, a mild developmental stress treatment influences adult beak color and induces adaptive phenotypic plasticity in endogenous antioxidant enzyme defenses, reducing oxidative damage in response to stressors.

Abbreviations:

ROS – reactive oxygen species

SOD – superoxide dismutase

GPx – glutathione peroxidase 1

4-HNE – 4-hydroxy-2-nonenal

CORT - corticosterone

Abstract

At certain intensities and durations, environmental stressors during development can result in changes in physiology that prepare organisms for future stressful conditions. Such plasticity can allow organisms to maintain good condition when confronted with a poor environment, potentially conferring an advantage in fitness. However, the physiological changes underlying these adaptive phenotypic adjustments are understudied. Using captive male zebra finches (*Taeniopygia castanotis)*, we tested whether exposure to a prolonged mild stressor during development would adaptively modify their antioxidant enzyme expression, reducing oxidative damage when exposed to a high intensity stressor in adulthood, and allowing the maintenance of a secondary sexual trait. To do this, we exposed juvenile finches to a prolonged mild heat stressor (38 °C) or control temperature (22 °C) treatment. As adults, these finches were then exposed to an acute high intensity heat stressor (42 °C) or control temperature (22 °C) treatment. The beak color of males – a sexually-selected trait – was quantified as well as oxidative stress parameters in the testes and liver tissues. We saw that mild heat conditioned males had beaks with higher saturation and lower brightness in adulthood, but the response to the high heat stressor varied. After exposure to the high heat stressor as adults, finches had higher levels of superoxide dismutase 1 & 2 in the testes and lower levels of lipid damage in the liver if they were also exposed to the mild heat conditioning as juveniles, indicating an adaptive phenotypic change.

Introduction

Environmental challenges experienced early in life can have persistent and irreversible effects on phenotype. Chronic stressors during development have been classically seen as detrimental due to their potential to suppress traits such as growth, immune function, and learning ability (Spencer et al., 2003; Harris and Seckl, 2011; Crino et al., 2014). However, chronic developmental stressors may not always be detrimental, with the direction of the effect depending upon the magnitude and timing of the exposure, as well as the adult environment. In accordance with the environmental matching hypothesis, developmental stressors can act instructively, resulting in phenotypic adjustments that make the organism more suited for the environment they are likely to experience in adulthood (Gluckman et al., 2005; Monaghan, 2008). For example, when these chronic developmental stressors are mild, it can result in an increased ability to tolerate higher levels of the stressor later in life, also known as conditioning hormesis (Calabrese et al., 2007; Costantini et al., 2010). However, if there is a mismatch between the early and later-life environments, such a change in phenotype may become detrimental (Monaghan, 2008). Most studies examining predictive adaptive responses utilize invertebrates as a study species and measure a limited number of response traits. Moreso, these studies rarely examine the changes in underlying physiology that act as mediators of the plasticity (Costantini et al., 2010).

Reproductive success is an important life-history trait that can be influenced by the developmental environment. One essential component of reproductive investment in many species is the production of secondary sexual traits, which in many species are assessed by females when choosing a mate (Andersson, 1994; Hill, 2006). For example, many avian species have beak and feather ornaments that derive their color from dietary pigments obtained through

the diet (Hill, 1999). These colorful ornaments can act as honest indicators of an individual's condition through their cost, which is the underlying principle of the costly signaling hypothesis (Zahavi, 1975; Weaver et al., 2017). In some iterations, the cost comes from having to trade-off resources between self-maintenance and ornament production, and therefore higher quality individuals will be able to afford to allocate more resources toward maintaining ornamentation (Zahavi 1975; Kodric-Brown and Brown, 1984; Grafen,,1990; Koch and Hill, 2018). As such, the environment and exposure to stressors may constrain these sexual traits by inducing trade-offs with functions related to self-maintenance and survival (Blount et al., 2003; Naguib and Nemitz, 2007; Butler and McGraw, 2011; Butler and McGraw, 2012). However, there are explanations for how honest signaling is maintained even without the presence of costs. In the index hypothesis, the honesty of signaling is not dependent on costs but rather because their production is tied to underlying physiological processes that cannot be "cheated" such as mitochondrial function and immune responses (reviewed in Weaver et al., 2017). Under both hypotheses, individuals with a higher tolerance to stressors should be able to better maintain ornaments when confronted with harsh conditions, either by avoiding costs and tradeoffs and/or by maintaining more optimal functioning of core physiological processes that determine condition.

Oxidative stress has been heavily implicated in the production and maintenance of colorful ornaments. Oxidative stress is generally described as an imbalance between reactive oxygen species (ROS) and the antioxidant defenses of an organism, eventually leading to an increase in oxidative damage to endogenous molecules (Finkel and Holbrook, 2000). Produced as a byproduct of normal aerobic cellular metabolism, ROS levels also increase with exposure to stressors, and can react with and damage lipids, proteins, and DNA (Halliwell and Gutteridge,

2015). Subsequently, accumulating oxidative damage can then lead to cellular senescence and reduced function of bodily systems over time (Finkel and Holbrook, 2000; Murphy et al., 2011). To counteract these detrimental effects, organisms produce antioxidant enzymes which react with and neutralize ROS to prevent oxidative damage (Finkel and Holbrook, 2000). Additionally, an organism can supplement its antioxidant levels through a diet which contains non-enzymatic antioxidant molecules, such as vitamin C & E and flavonoids (Halliwell and Gutteridge, 2015). Carotenoids are another such molecule, which are pigments derived from plants that provide the red-orange color for many avian ornaments (Lucas and Stettenheim, 1972; Hill, 1999). However, for carotenoids, their antioxidative function *in vivo* is under debate (Costantini and Møller, 2008). An organism's antioxidant defense system can also be primed via exposure to mild oxidative stress, by upregulating production of antioxidant enzymes which protects against more severe oxidative insults later. For example, when *E. coli* and many other bacteria are exposed to mild levels of hydrogen peroxide (a reactive oxygen species), they show greater resistance to higher levels of hydrogen peroxide during a subsequent exposure (Faulkner and Helmann, 2011). Therefore, prior experiences can influence the function of processes that determine individual condition, that may be reflected by the status of colorful ornaments (Hill, 2011).

The antioxidant system in vertebrates is highly plastic, and a significant amount of variation in an individuals' ability to resist oxidative stress can be explained by the conditions experienced during development (Costantini, 2014). Exposure to mild stressors during early life may cause an alteration in antioxidant enzyme production which persists into adulthood. If so, organisms with antioxidant defenses previously attuned to non-optimal environments may forgo trade-offs and avoid diminishment of sexually selected traits later in life. Specifically, in birds, it

has been shown that adult carotenoid-based traits and the antioxidant defense system can be influenced by early life conditions (Noguera et al., 2015; Romero-Haro and Alonso-Alvarez, 2015; Romero-Haro et al., 2016). For example, zebra finches (*Taeniopygia castanotis*) given a diet with reduced antioxidant vitamins and minerals were able to adjust their endogenous production of antioxidants to compensate, and were able to maintain elevated total antioxidant capacity and glutathione peroxidase (GPx) into adulthood when kept on the same diet (Noguera et al., 2015). On the other hand, when there was a mismatch between early (0-40 days post-hatch) and late development (40-90 days post-hatch) diet, components of the adult antioxidant defense system were diminished (Noguera et al., 2015). Another study found that zebra finches with experimentally decreased glutathione during development had redder beaks as adults, but also suffered increased oxidative damage (Romero-Haro and Alonso-Alvarez, 2015). However, it is still unclear as to what extent the expression of a carotenoid-based trait may indicate antioxidant enzyme levels, and whether adult antioxidant synthesis can be adaptively modified by developmental conditions in a hormetic manner.

In this study, we used captive zebra finches to test the hypothesis that a prolonged mild thermal stressor during the juvenile period acts in a conditioning hormetic manner, resulting in altered antioxidant enzyme synthesis that persists into adulthood. We also hypothesized that this potential upregulation of antioxidants is associated with an increased protection against oxidative damage and the attenuation of a secondary sexual trait – beak coloration – when exposed to a high intensity thermal stressor as adults. Like many avian species, zebra finches have vascularized red-orange beaks that get their color from carotenoids (Lucas and Stettenheim, 1972). Beak color is a sexually selected trait in zebra finches, with some evidence indicating that females prefer males with redder beaks (Coopersmith, 1987; Collins and Ten Cate, 1996;

McGraw et al., 2002; Blount et al., 2003; but see Forstmeier and Birkhead, 2004). One study has shown that the color of zebra finch beaks can be diminished by exposure to thermal stress, and another has demonstrated that they are able to change color with circulating levels of carotenoids as quickly as within three days (Eraud et al., 2007; Ardia et al., 2010). As such, we exposed juvenile (starting 42-45 days post-hatch) zebra finches to a mild prolonged heat stressor (38 °C) or control temperature (22 °C). Then as adults (170-173 days post-hatch), we exposed these individuals to either a high heat stressor (42 °C) or control temperature (22 °C) in a 2x2 factorial design. We predicted that finches that received the mild heat conditioning as juveniles would have higher antioxidant enzyme levels in liver and testes tissue, more colorful beaks, and lower levels of tissue oxidative damage when exposed to a high heat stressor as adults, compared to those that did not receive the mild heat conditioning.

Materials and Methods

Thermal treatments

For a full description of the general husbandry methods and thermal treatment procedures, see our previously published manuscripts (Hoffman et al., 2018; Hoffman et al., 2023). In brief, juvenile male (n = 23 zebra finches at 42-45 days post-hatch underwent a mild heat conditioning of 38 °C for 3 hours per day in a brooder (TLC-50, Brinsea, Florida), every other day for 28 days following Costantini et al. (2012). The other juveniles served as a control group (n=17) and were exposed to the room temperature (21-23 °C) in the same fashion. The 38 °C treatment can be considered a mild heat stressor because the temperature is just outside the upper critical limit of the thermoneutral zone (TNZ) for this species as determined by Wojciechowski et al. (2021; but see also Calder, 1964), and it has been shown that the protocol does not increase oxidative damage in blood plasma (Costantini et al., 2012). In a 2x2 factorial

design, half of the males from each juvenile treatment group were exposed to high heat of 42 °C or control (21-23 °C) temperature for 3 hours per day for 3 consecutive days at 170-173 days post-hatch in brooders as described above. This 42 °C treatment can be considered a high heat stressor as the temperature is considerably above the TNZ for the species (Wojciechowski et al., 2021), and this specific protocol has been previously demonstrated to result in an increase in plasma oxidative damage (Costantini et al., 2012). This procedure resulted in four distinct treatment groups that represent the different combinations of juvenile and adult treatment [Control-Control (CC): n = 8, Control-Heat (CH): n = 10, Mild-Control (MC): n = 13, Mild-High (MH): n = 10].

Beak color quantification

Beak coloration was determined using digital photographs taken on the day prior to (Day 0) and on the final day (Day 3) of adult stage treatment. In order to standardize conditions across days, the birds were held in a photography light tent (LimoStudio 16”x16” Photography Light Tent Kit, AGG349) while the photographs were taken. Two 120V 50W lamps were placed on each side of the tent at fixed positions to provide light. Photographs were taken using a digital camera (Fujifilm FinePix F30) placed on a small tripod, with the camera settings placed to manual. Males were held and oriented to get a lateral view of the beak, and a photograph of the right side of the maxilla was then taken for each individual. The digital images were then uploaded to ImageJ and the beak area was selected using the polygon selection tool. The beak color was analyzed using the Plugins>Analyze>RGB measure function. From this, RGB (Red, Green, Blue) values were obtained, which were then used to calculate values for hue, saturation, and brightness using a conversion tool previously validated in our lab (Choi et al., 2022). Some

photographs were not used due to the quality of images and bird position. Thus, the final sample size for the beak color quantification was CC: n = 6, CH: n = 9, MC: n = 11, MH: n = 9.

Tissue collection and quantification of proteins using Western blot

At the end of the three-day adult treatment period, males (N=40) were euthanized via isoflurane inhalation and testes and liver tissues were dissected approximately 4 hours following the final 3-hour treatment bout. Tissues were weighed and then flash frozen in liquid nitrogen before being stored at -80 °C. Western blots were performed on the left lobe of the liver and both testes, and samples were homogenized and prepped for gel electrophoresis and blotting as previously described (Hyatt et al., 2016). The levels of tissue antioxidant enzymes superoxide dismutase 1 (SOD-1, GTX100554, GeneTex), superoxide dismutase 2 (SOD-2; GTX116093, GeneTex) and glutathione peroxidase 1 (GPX1; GTX116040, GeneTex), as well as a lipid peroxidation marker 4-hydroxy-2-nonenal (4-HNE, GTX17571, GeneTex) were quantified by Western blot as previously described (Finger et al., 2017, 2018, 2021). Protein oxidation levels were determined by measuring protein carbonyls using a commercially available kit (OxyBlot; s7150; EMD Millipore, Billerica, MA, USA) via Western Blot as described by manufacturer's instructions (Yap et al., 2022). The membranes were stained using Ponceau stain to act as a loading and transfer control and imaged using a chemiluminescent system (ChemiDocIt Imaging System (UVP, LLC, Upland, CA, USA).

Statistical analyses

All statistical analysis was done in RStudio (RStudio Team, 2020) using the package "nlme" and graphs were created using the "ggplot2" package. Tables with statistical model outputs were made using the "tab_model" function in the package "sjPlot". We used linear

mixed-effects models (LMM) to determine the effect of juvenile and adult treatments on beak color and all oxidative status parameters. Pearson correlation tests were used to examine the relationships between beak color parameters. Statistical significance was determined as $P \leq 0.05$, and all models included the nest ID as a random effect to account for multiple males originating from the same genetic nest. Models were constructed in a backward stepwise manner, starting with the full model, predictors were removed when $P \geq 1.00$ one at a time. Assumptions of linearity of the data were tested by plotting the fitted values versus residuals. Assumptions of normality of residuals were tested via histogram plots of the data for linear models. Regression coefficients were used for estimates of mean change in response variables for linear models. Estimates of uncertainty were represented via 95% confidence intervals (CI), which were determined using the "intervals" package and function for linear regressions.

Results

Testes antioxidant enzymes and oxidative damage

When examining levels of SOD-1 found in the testes tissue we saw a significant juvenile*adult (J*A) treatment interaction (LMM: J*A: $\beta = 2.57$, $t = 3.19$, d.f. = 10, $P = 0.010$). Among unconditioned finches, those exposed to the high heat stressor treatment in adulthood had SOD-1 levels 1.55 AU ([-2.93, -0.177], ±95% CI) lower than finches exposed to the control treatment as adults (Fig. 1A) (LMM: CC – CH: $t = -2.52$, d.f. = 10, $P = 0.031$). However, finches that underwent the mild heat conditioning as juveniles did not exhibit this decrease in SOD-1 levels in response to the high heat stressor as adults, and had levels 1.63 AU ([0.430, 2.83], ±95% CI) higher than the unconditioned finches (Fig. 1A) (LMM: CH-MH: $t = 3.03$, d.f. = 10, $P = 0.013$). When looking at SOD-2 levels in the testes we again observed a similar conditioning hormetic effect. Finches that were not conditioned as juveniles but were exposed to the high heat

stressor as adults had lower levels of SOD-2 in the testes than all other groups, including the conditioned-high heat adults (Fig. 1B) (LMMs: CC-CH: β = 1.83, *t* = 3.02, d.f. = 13, *P* = 0.0099; MC-CH: β = 1.30, *t* = 2.43, d.f. = 13, *P* = 0.031; CH-MH: β = 1.69, *t* = 3.00, d.f. = 13, *P* = 0.010).

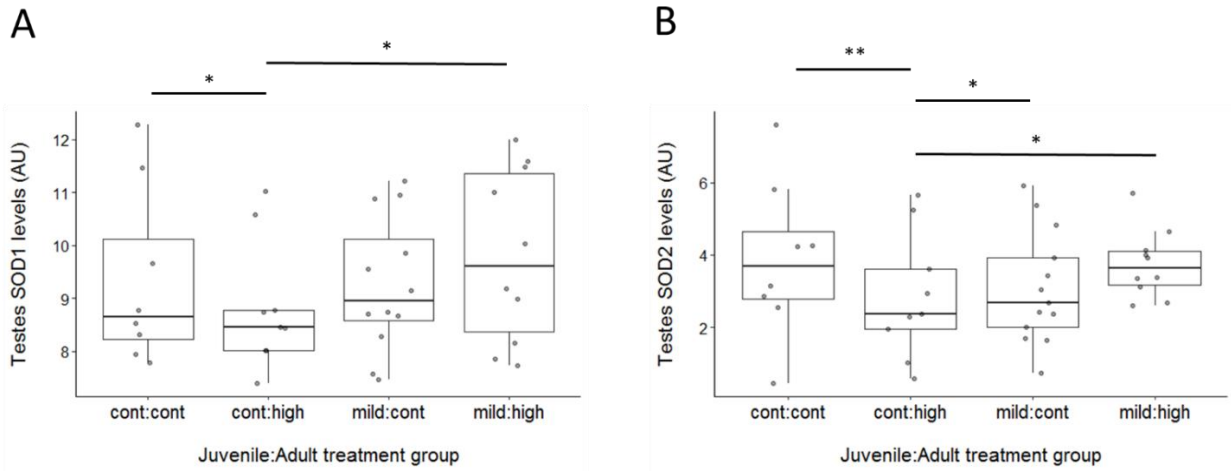

Fig. 1. Effects of juvenile and adult thermal treatments on testes superoxide dismutase (SOD) levels following the adult treatment period (173 DPH). (A) After exposure to the high heat stressor (42 °C) as adults, finches that were exposed to the control temperature (22 °C) treatment as juveniles (Control-High) had lower levels of SOD-1 in their testes compared to the conditioned counterparts (Mild-High), as well as finches exposed to the control temperature at both timepoints (Control-Control). **(B)** After exposure to the high heat stressor (42 °C) as adults, finches exposed to the control temperature treatment (22 °C) as juveniles (Control-High) had lower levels of SOD-2 in their testes compared to all other groups. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

When looking at GPx levels in the testes we saw a stimulatory and additive effect of heat treatments. Finches exposed to either the mild heat conditioning as juveniles, the high heat stressor treatment as adults, or both, had higher levels of GPx compared to those never exposed to a heat treatment at any point (Fig. 2A) (LMMs: CC-CH: β = 1.29, *t* = 2.35, d.f. = 15, *P* = 0.033; CC-MC: β = 1.55, *t* = 3.03, d.f. = 15, *P* = 0.0084; CC-MH: β = 2.42, *t* = 4.58, d.f. = 15, *P* = 0.0004). Oxidative damage in the testes was measured by quantifying the presence of protein carbonyls, a byproduct of protein oxidation. When examining the levels of protein carbonyls in

the testes, we saw no substantial effect of the juvenile treatment, adult treatment, or their interaction (Fig. 2B) (LMM: Juvenile: β = 0.926, *t* = 1.68, d.f. = 13, *P* = 0.118; Adult: β = 0.428, *t* = 0.742, d.f. = 13, *P* = 0.471; J*A: β = -0.923, *t* = -1.18, d.f. = 13, *P* = 0.26).

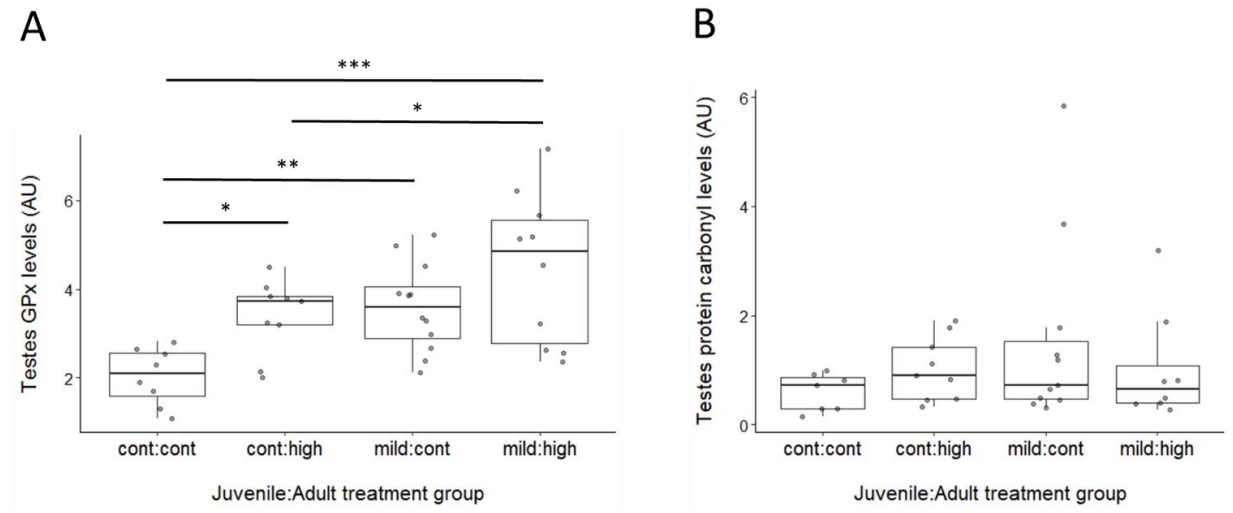

Fig. 2. Effects of juvenile and adult thermal treatments on testes glutathione peroxidase-1 (GPx) and protein oxidative damage (protein carbonyls) following the adult treatment period (173 DPH). (A) Finches that were exposed to the control temperature treatment (22 °C) at both the juvenile and adult treatment timepoints (Control-Control) had lower levels of GPx in the testes compared to all other groups. **(B)** There was no significant effect of juvenile or adult treatment on testes protein carbonyl levels. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Liver antioxidant enzymes and oxidative damage

We also measured antioxidant enzyme levels in liver tissue of the finches following the 3-day adult treatment. When examining levels of SOD-1 in the liver, we saw that the finches exposed to the mild heat conditioning as juveniles and the control treatment as adults had 0.0964 AU ([0.0074, 0.185], ±95% CI) less SOD-1 enzymes than non-conditioned birds that were exposed to the high heat treatment in adulthood (Fig. 3A) (LMM: MC-CH: *t* = 2.31, d.f. = 15, *P* = 0.036). However, when examining SOD-2 levels in the liver we saw a stimulatory effect of

heat treatments rather than the expected pattern of conditioning hormesis. Finches exposed to an elevated temperature – either the juvenile mild heat conditioning, adult high heat treatment, or both, had higher levels of SOD-2 in the liver than those never exposed to any thermal stressor (Fig. 3B) (LMMs: CC-CH: $\beta = 0.181$, $t = 3.84$, d.f. = 13, $P = 0.0020$; CC-MC: $\beta = 0.131$, $t = 3.28$, d.f. = 13, $P = 0.0060$; CC-MH: $\beta = 0.137$, $t = 3.41$, d.f. = 13, $P = 0.0046$).

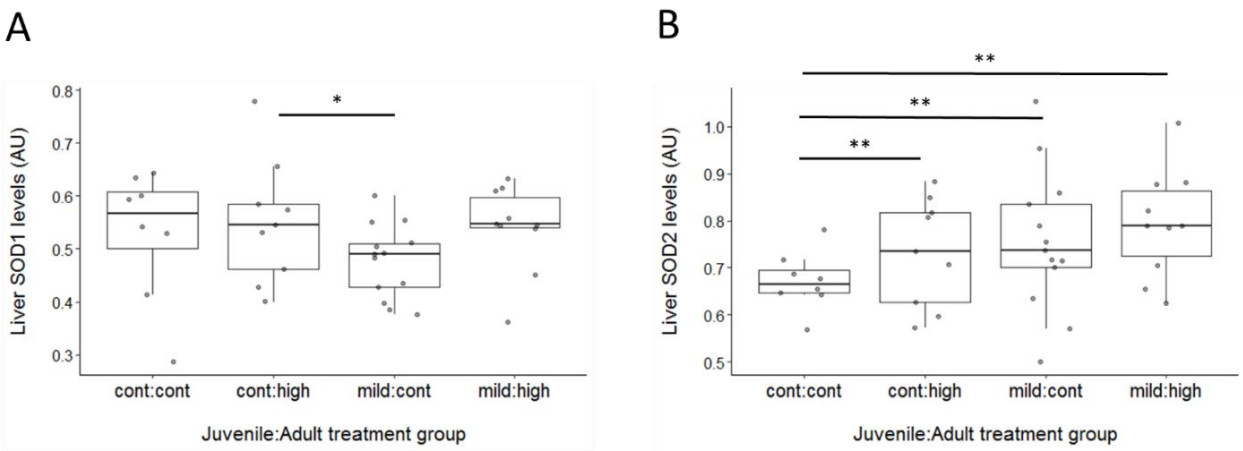

Fig. 3. Effects of juvenile and adult thermal treatments on liver superoxide dismutase (SOD) levels following the adult treatment period (173 DPH). (A) Non-conditioned finches that were later exposed to the high heat stressor as adults (Control-High) had higher levels of SOD-1 in their liver compared to the birds that were exposed to the mild heat conditioning as juveniles, but the control treatment as adults (Mild-Control). **(B)** Finches that were exposed to the control temperature treatment (22 °C) as both the juveniles and adults (Control-Control) had lower levels of SOD-2 in their liver compared to all other groups. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

With regards to GPx levels in the liver, we once again saw a seemingly additive effect of heat exposure. Following the 3-day adult treatment period, we saw that finches exposed to the mild heat conditioning as juveniles and the high heat stressor as adult had higher GPx levels than all other groups (Fig. 4A) (LMMs: MH-CC: $\beta = 0.135$, $t = 2.78$, d.f. = 11, $P = 0.018$; MH-CH: $\beta = 0.102$, $t = 2.44$, d.f. = 11, $P = 0.033$; MH-MC: $\beta = 0.094$, $t = 2.39$, d.f. = 11, $P = 0.036$). Oxidative damage in the liver was measured by quantifying the presence of 4-HNE, a byproduct

of lipid oxidation. As expected, exposure to the 3-day high heat treatment as adults increased levels of 4-HNE in the liver, as indicated by the comparison of the cont:cont and cont:high groups (Fig. 4B) (LMM: CC-CH: β = -0.0146, *t* = -2.58, d.f. = 12, *P* = 0.024). When examining 4-HNE levels we saw the predicted conditioning hormetic pattern, where finches exposed to the mild heat conditioning as juveniles had significantly lower 4-HNE levels after exposure to the high heat treatment as adults, compared to their juvenile control counterparts (Fig. 4B) (LMM: CH-MH: β = -0.0124, *t* = -2.25, d.f. = 12, *P* = 0.044), and similar levels to the Control-Control and Mild-Control groups (Fig. 4B) (LMMs: CC-MH: β = -0.0228, *t* = -0.426, d.f. = 12, *P* = 0.678; MC-MH: β = -0.00585, *t* = -1.16 , d.f. = 12, *P* = 0.269).

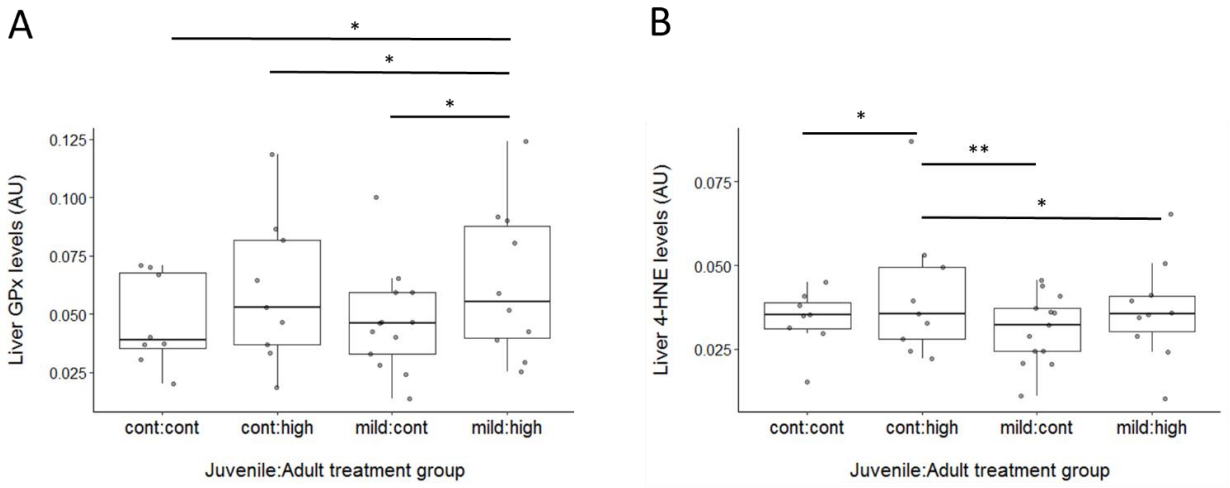

Fig. 4. Effects of juvenile and adult thermal treatments on liver glutathione peroxidase-1 (GPx) and lipid oxidative damage (4-hydroxynonenal – 4HNE) following the adult treatment period (173 DPH). (A) Finches exposed to both the mild heat conditioning (38 °C) as juveniles and the high heat stressor (42 °C) treatment as adults had higher levels of GPx in the liver compared to all other groups. **(B)** After exposure to the high heat stressor (42 °C) as adults, finches exposed to the control temperature treatment (22 °C) as juveniles (Control-High) had higher levels of 4-HNE in the liver compared to all other groups. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

Relationships between antioxidant enzymes and oxidative damage

We also examined the relationships between different antioxidant enzymes and damage parameters. In the liver, we observed the expected negative relationship when looking at one antioxidant and lipid oxidation. For each 1 unit (AU) increase in liver SOD-1 levels, we saw a 5.04 $x10^{-2}$ AU ([-9.26 $x10^{-2}$, -8.23 $x10^{-3}$], ±95% CI) decrease in 4-HNE in the liver (LMM: t = -2.60, d.f. = 12, P = 0.0233). Interestingly, we saw a positive relationship between SOD-1 in the testes and 4-HNE (lipid damage) in the liver. For each 1 unit (AU) increase in testes SOD-1 levels, we saw a 2.96 $x10^{-3}$ AU ([2.77 $x10^{-5}$, 5.90 $x10^{-3}$], ±95% CI) increase in 4-HNE in the liver (LMM: t = 2.20, d.f. = 12, P = 0.0482). We saw the same pattern with regards to the relationship between SOD-2 in the testes and 4-HNE (lipid damage) in the liver. For each 1 unit (AU) increase in testes SOD-2 levels, we saw a 4.58 $x10^{-3}$ AU ([1.97 $x10^{-4}$, 7.20 $x10^{-3}$], ±95% CI) increase in 4-HNE in the liver (LMM: t = 3.83, d.f. = 12, P = 0.0024). Contrary to expectations, we saw no significant relationship between testes antioxidant enzyme levels and the amount of testes protein oxidation (protein carbonyls).

The effect of juvenile and adult thermal treatment on beak color

We observed no significant effect of the juvenile treatment on the hue of adult beak color prior to the start of the adult treatment (Hue: t = -0.37, d.f. = 14, P = 0.54). However, we saw that male finches exposed to the mild heat treatment as juvenile had beaks with 5.53% ([1.40, 9.65] ±95% CI) higher color saturation and 4.84% ([-9.15, -0.53], ±95% CI) lower brightness (value compared to the juvenile control finches (Fig. 5A & Fig. 6A)(LMMs: Saturation: t = 2.87, d.f. = 14, P = 0.012; Brightness: t = -2.41, d.f. = 14, P = 0.031). We saw that after the 3 day adult treatment period, finches exposed to either the mild heat conditioning as juveniles, high heat stress as adults, or both, had less saturated beaks than those that received the control treatment at

both time points (Fig. 5B) (LMMs: CC-CH: β = -10.3, t = -3.87, P = 0.008; CC-MC: β = -7.62, t = -3.67, P = 0.010; CC-MH: β = -7.27, t = -3.12, P = 0.021). Furthermore, finches that were exposed to the high heat stressor as adults – but not the mild heat conditioning as juveniles (cont:high) – had the largest decrease in beak saturation, suffering a 6.73% ([0.22, 13.2], ±95% CI) larger decrease in saturation in comparison to the mild:cont group (LMM: t = 2.25, d.f. = 12, P = 0.044).

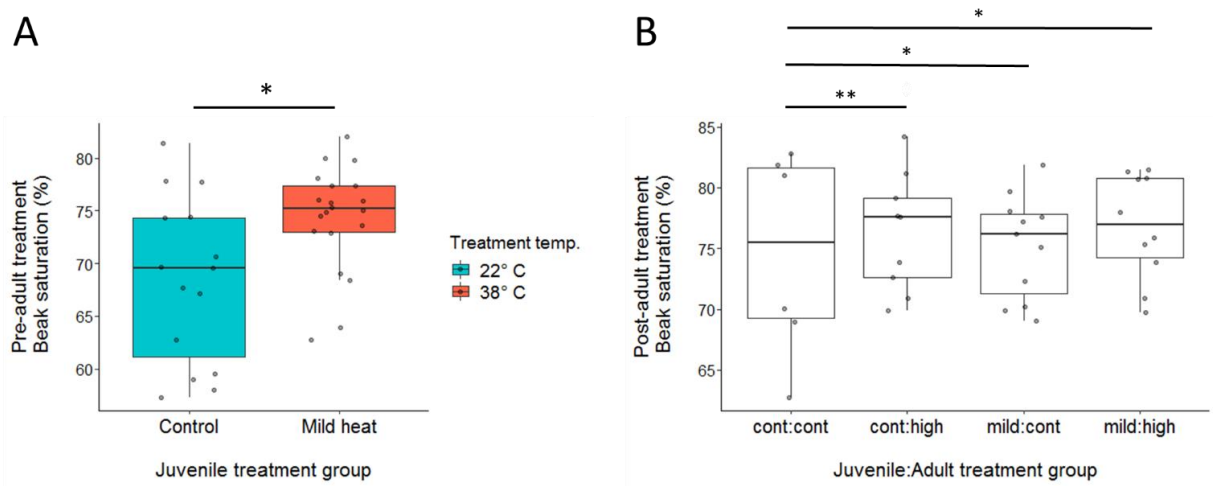

Fig. 5. Effects of thermal treatments on beak saturation. (A) Male zebra finches exposed to the mild heat conditioning treatment as juveniles had beaks with higher saturation as adults (170 DPH). The legend denotes the different temperature each group was exposed to as juveniles, either a control (22 °C) or mild heat conditioning (38 °C). **(B)** Following the adult treatment period, finches that were exposed to the control temperature treatment 22 °C at both timepoints (Control-Control), had higher beak saturation than all other groups. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

We also observed that the adult high heat stressor treatment affected beak brightness, however, only when finches had prior heat exposure. After the 3-day adult treatment period, finches that were exposed to the high heat stressor and were also exposed to the mild heat conditioning as juveniles had 9.39% ([-18.1, -0.66], ±95% CI) lower beak brightness that those exposed to the control treatment at both timepoints (Fig. 6B) (LMM: CC – MH: t = -2.43, d.f. =

9, P = 0.038). As expected, these mild:high finches had a substantially larger increase in saturation across the 3-day period compared to the cont:cont and mild:cont groups (LMMs: CC – MH: β = -10.5, t = -3.00, d.f. = 10, P = 0.014; MC – MH: β = -8.21, t = -2.70, d.f. = 10, P = 0.022). We observed no differences in beak hue due to the juvenile or adult treatments following the 3-day adult treatment period (LMM: Juvenile: β = -1.69, t = -1.95, d.f. = 9, P = 0.084; Adult: β = -0.83, t = -0.95, d.f. = 9, P = 0.37; J*A: β = 2.23, t = 1.81, d.f. = 9, P = 0.104) nor significant differences in the change in hue across this period (LMM: Juvenile: β = -0.22, t = -0.43, d.f. = 11, P = 0.68; Adult: β = -0.39, t = -0.71, d.f. = 11, P = 0.49; J*A: β = -0.071, t = -0.10, d.f. = 11, P = 0.92).

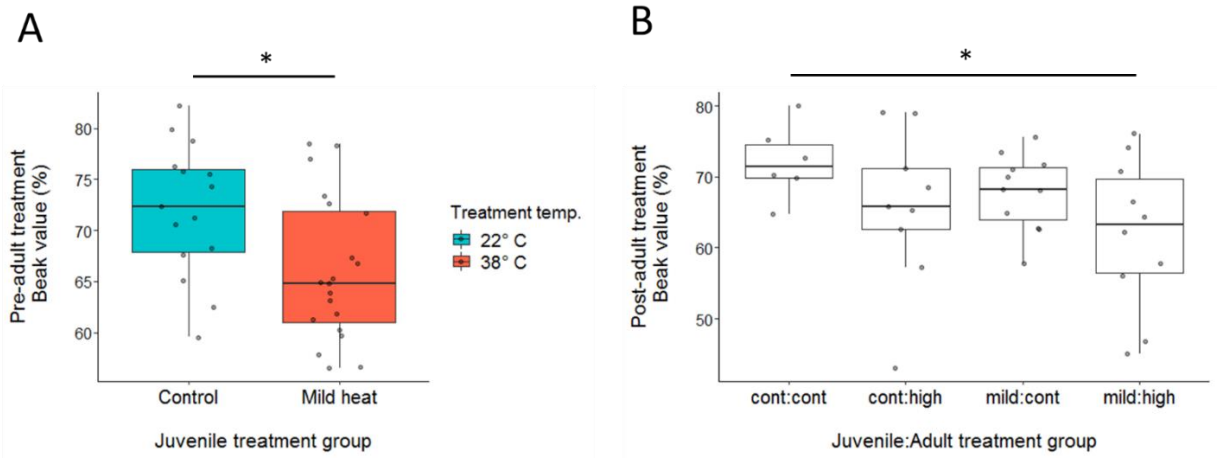

Fig. 6. Effects of thermal treatments on beak value/brightness. (A) Male zebra finches exposed to the mild heat conditioning treatment as juveniles had beaks with lower value/brightness as adults (170 DPH). The legend denotes the different temperature each group was exposed to as juveniles, either a control (22 °C) or mild heat conditioning (38 °C). **(B)** Males exposed to both the mild heat conditioning (38 °C) as juveniles and the high heat stressor (42 °C) treatment as adults had lower beak value/brightness than the Control-Control group after the adult treatment period. For each group on the x-axis, the first word denotes the treatment the males received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Relationships between oxidative state and beak color

We saw a significant relationship between post-adult treatment beak hue and testes SOD-1 levels, however it depended on the treatment birds were exposed to. In the cont:cont group, for each 1 unit (AU) increase in testes SOD-1 levels, we saw a 1.065 degree ([0.633, 1.50], ±95% CI) increase in beak hue (LMM: t = 6.84, d.f. = 4, P = 0.0024). This positive relationship in the cont:cont group was significantly different than the weak correlation between testes SOD-1 and beak hue seen in the other groups (LMMs: CC-CH: β = -0.967, t = -3.84, d.f. = 4, P = 0.0185; CC-MC: β = -1.29, t = -5.41 , d.f. = 4, P = 0.0057; CC-MH: β = -0.988, t = -7.15 , d.f. = 4, P = 0.0020). We also saw a significant relationship between post-adult treatment beak saturation and testes SOD-2 levels, however it again was dependent on the treatment birds were exposed to. In the cont:cont group, for each 1 unit (AU) increase in testes SOD-2 levels, we saw a 2.43 % ([-4.84, -0.0100], ±95% CI) decrease in beak saturation (LMM: t = -2.46 d.f. = 6, P = 0.0493). This negative relationship between beak saturation and testes SOD-2 levels seen in the cont:cont group was in significant contrast to that seen in the mild:high group, which displayed a positive relationship between the two variables (LMM: β = 4.82, t = 2.90, d.f. = 6, P = 0.0275). When examining the relationship between beak brightness and testes SOD-2 levels, we saw a difference between groups, but it was dependent upon the treatment birds were exposed to as adults. When exposed to the high heat stressor as adults, birds exhibited a positive relationship between beak brightness and testes SOD-2 levels if they were also exposed to the mild heat conditioning as juveniles. In the mild:high group, for each 1 unit (AU) increase in testes SOD-2 levels, we saw a 11.54 percent ([3.57, 19.5], ±95% CI) increase in beak hue (LMM: t = 3.54, d.f. = 6, P = 0.0122), which was significantly different than the relationship demonstrated by all

other groups (LMMs: MH-CC: β = -11.09, *t* = -3.02, d.f. = 6, *P* = 0.0234; MH-CH: β = -13.6, *t* = -3.66 , d.f. = 6, *P* = 0.0105; MH-MC: β = -13.6, *t* = -3.96 , d.f. = 6, *P* = 0.0074).

Relationships among beak color parameters

We examined the relationships among the hue, saturation, and brightness both before and after the adult treatment period. Prior to the adult treatment (170 days post-hatch), we saw a significant positive correlation between the hue and brightness of beaks (*r* = 0.406, [0.0839, 0.651] ±95% CI, *t* = 2.55, d.f. = 33, *P* = 0.0156), however, the hue was not correlated with beak saturation (*r* = 0.0778, [-0.262, 0.401] ±95% CI, *t* = 0.448, d.f. = 33, *P* = 0.657). At this timepoint we also saw a significant relationship between beak saturation and brightness, but in this case it was a negative correlation (*r* = -0.439, [-0.674, -0.124] ±95% CI, *t* = -2.81, d.f. = 33, *P* = 0.00829). When looking at the relationships between beak color parameters following the adult treatment, we again saw a significant positive correlation between the hue and brightness of beaks (*r* = 0.463, [0.158, 0.687] ±95% CI, *t* = 3.045, d.f. = 34, *P* = 0.00447), and no strong correlation between beak hue and saturation (*r* = -0.459, [-0.397, 0.257] ±95% CI, *t* = -0.459, d.f. = 34, *P* = 0.649). In contrast with before the adult treatment period, there was only a weak and nonsignificant correlation between beak saturation and brightness following this treatment period (*r* = -0.278, [-0.556, 0.0558] ±95% CI, *t* = -1.69, d.f. = 34, *P* = 0.101).

Discussion

The main goal of this study was to determine if a mild heat conditioning during the juvenile period adaptively alters antioxidant enzyme production, allowing maintenance of a sexually selective trait and reduced oxidative damage when faced with a stressor in adulthood. We saw in both the testes and liver tissue that mild heat exposure during development altered

antioxidant enzyme levels, but the effect often depended on whether the birds were exposed to a high heat stressor as adults. In the testes, when looking at SOD-1 and SOD-2 antioxidant enzymes, we saw a pattern indicative of conditioning hormetic effects. Heat-conditioned males that experienced the high heat stressor as adults (mild:high) had an equally high SOD-1&2 levels in the testes compared to males never exposed to heat (cont:cont). However, when males were exposed to the high heat stressor as adults without previously undergoing the conditioning as juveniles (cont:high), they had lower levels of SOD-1&2 in the testes compared to mild:high and cont:cont individuals. These results indicate that the conditioned group either had an altered antioxidant response more attuned to dealing with a high heat stressor, or were in a state of lower oxidative stress due to increased resistance as the result of other physiological mechanisms. However, not all antioxidant enzyme parameters measured followed this conditioning hormesis pattern. In the testes, there instead seemed to be a stimulatory effect of heat on GPx production, with the cont:cont males having lower levels of this antioxidant enzyme than all other groups. The effect of heat was also additive, as the individuals that were exposed to the mild and high heat stressors (mild:high) had higher levels of GPx in the testes than those exposed to only the high heat as adults (cont:high). When examining oxidative damage levels in the testes tissue – measured via protein carbonyls – we observed no effects of treatment. While this result is contrary to our prediction, it may not be surprising. In testes tissue, spermatogenesis is a significant source of ROS production, and avian sperm are generally considered at a higher risk of oxidative damage. This is due to their high amounts of unsaturated fatty acid relative to other tissues, and their low DNA repair capacity (Surai, 1998). Increased oxidative damage in the testes can negatively impact reproduction, and is thought to be a significant contributor to fertility issues in male chickens (Wishart, 1984). Therefore, due to the increased likelihood for

oxidative damage in the testes to have substantial impacts on fitness, finches may invest more into testes antioxidant defenses, making them more resistance to stressor-induced damage.

In the liver tissue we measured the same endogenous antioxidant enzymes as in the testes, but we observed a different overall pattern. Contrary to the hormetic pattern observed in the testes, we saw a potential cost of the mild heat conditioning with regard to SOD-1 levels in the liver. The males exposed to the conditioning as juveniles but not the high heat as adults (mild:cont) demonstrated lower SOD-1 levels than cont:cont males. When examining SOD-2 levels, we saw a seemingly stimulatory effect of heat on levels in the liver, akin to a hormetic effect in the traditional sense often used in toxicology. The males that were never exposed to any heat treatment (cont:cont) had lower levels of SOD-2 in the liver than all other groups. When measuring GPx, we saw a seemingly additive effect of heat exposure. The males exposed to the mild heat conditioning as juveniles and high heat stressor in adulthood (mild:high) had higher levels of GPx in the liver compared to all the other groups. In order to accurately gauge an organism's level of oxidative stress, it's invaluable to measure damage parameters, as antioxidant levels alone paint an incomplete and sometimes deceptive picture. When looking at the levels of 4-HNE – a byproduct of lipid peroxidation – in the liver, we saw a pattern consistent with conditioning hormetic effects. Exposure to the high heat stress as adults resulted in an increase in lipid damage, with the males that received no heat conditioning as juveniles (cont:high) incurring higher levels of 4-HNE than control individuals (cont:cont). However, if the males were exposed to the mild heat conditioning as juveniles, they had lower levels of 4-HNE after exposure to the high heat stressor treatment as adults. This group (mild:high) also had levels of 4-HNE similar to those never exposed to any heat treatments (cont:cont). Together

these results indicate that the mild heat conditioning resulted in a phenotypic adjustment which primed male birds to be more resistant to oxidative stress when exposed to a subsequent stressor.

While the heterogeneity in antioxidant enzyme levels both within and between tissues was not predicted, it is also not entirely surprising. Sampling from multiple tissue types has the benefit of giving a more comprehensive view of an individual's oxidative status, but there are also drawbacks to this approach. Often this approach means sampling tissues at a single time point, but different tissues may have different response times with regards to antioxidant mobilization or incurrence of damage when exposed to perturbations (Costantini, 2019). This is not only because different tissues vary in antioxidant production capabilities and damage repair, but also in antioxidant gene expression (Halliwell and Gutteridge, 2015; Wu et al., 2015). This temporal variation may lead to a single sampling point creating a disingenuous picture of the true effects of a stimulus on an individual's oxidative status. The heterogeneity in antioxidant enzyme levels between tissue types may also be explained in part by the structure of the tissues themselves. Macromolecules vary in their susceptibility to oxidative damage due to their chemical components and structure (Pamplona and Barja, 2007). For example, unsaturated fatty acids found in cells membranes are more susceptible to oxidative damage than monounsaturated and saturated counterparts (Hulbert et al., 2007; Pamplona and Costantini, 2011). Therefore, because susceptibility of tissues to oxidative damage can vary due to their cellular structure, it is plausible that the antioxidant enzyme production levels vary in response, and contribute to the variation seen between tissues and across species.

Tissue heterogeneity in antioxidant enzyme levels may also be a consequence of prioritization of some tissues with regards to preventing oxidative damage, depending on their correlation with overall fitness. For example, damage to the testes and sperm can directly

influence the number of offspring sired, so their protection may be given priority and come at a cost to tissues such as the liver. Perhaps in support of this, we saw here that across all samples, higher levels of SOD-1 and SOD-2 in the testes were associated with higher levels of lipid damage (4-HNE) in the liver. On the other hand, there was also the expected relationship between SOD-1 and 4-HNE levels in the liver, where higher levels of SOD-1 were associated with lower levels of 4-HNE. Interestingly, this relationship was only significant when testes SOD levels were also accounted for in the statistical model. These dependent relationships between antioxidant enzymes both within and across tissues may also be due in part to their coordinative nature. For example, the SODs are often considered the "first line" of defense against ROS, speeding up dismutation to produce diatomic oxygen and hydrogen peroxide (Halliwell and Gutteridge, 2015). While hydrogen peroxide is relatively much less reactive than superoxide, it can also induce oxidative damage and must be kept under control (Halliwell and Gutteridge, 2015). This is where the "second level" defenses such as GPx come into play, reducing hydrogen peroxide to water. This coordinated and tiered structure of the antioxidant defense network is also important as it prevents ROS from damaging the antioxidant enzymes themselves. For example, mice without SOD-1 had lower GPx1 activity in liver tissue because the selenocysteine – an essential component of proper GPx1 catalysis action – was converted to a different molecule as a result of increased ROS exposure (Wang et al., 2011). For these reasons, many urge caution when trying to make conclusions regarding the oxidative status of the entire organism based on a single antioxidant parameter and tissue type.

To determine how a sexually-selected ornament was affected by our treatments, we measured three parameters of beak coloration in male zebra finches. Hue is the most commonly measured parameter and can be thought of as the characteristic distinguishing colors such as red,

yellow, and blue from one another. Most of the research on beak coloration in zebra finches has focused on hue, indicating that females prefer mates with lower hues (redder beaks) (Coopersmith, 1987; Collins & Ten Cate, 1996; McGraw et al., 2002; Blount et al., 2003). In our study we saw no effect of the juvenile or adult treatment on the hue of male beaks. While we have shown that the 3-day high heat stress as adults was of a high enough magnitude to change antioxidant levels and inflict lipid oxidative damage, it is possible that the high heat treatment itself and/or time of measurement (of hue) was too short to capture a change in this higher-level trait. To our knowledge, the only study to show a change in beak hue in this species within a 3-day period used exogenous testosterone as their treatment (Ardia et al., 2010). Such a treatment potentially has the capability for more immediate and influential effects, compared to a more ecologically relevant heat stress treatment.

Another parameter we measured was the saturation – or intensity – of beak color. Contrary to our predictions, the change in beak color saturation did not follow the expected hormetic pattern observed in some of the antioxidant and damage parameters. We observed that males exposed to any heat at either treatment period (mild, high, or both) had lower beak color saturation than those never exposed to heat (cont:cont) following the adult treatment period. Although it was trending toward the expected pattern, as the mild:high group had marginally higher saturation than the cont:high group, it is possible that with a higher sample size we would see the expected differences between those two groups of males. The third color parameter we measured was the value – or brightness – of beaks. To our knowledge, there is no literature indicating female preference with regards to the brightness of beak color in zebra finches. However, we did see in our study that across all samples, brightness was positively correlated with hue (lower number = redder beaks), and lower brightness numbers generally correspond

with redder beaks richer in carotenoids (Merrill et al., 2016). We also observed that the males exposed to both the heat conditioning as juveniles and the high heat stressor as adults (mild:high) had lower beak brightness as adults than those never exposed to heat (cont:cont).

It is unclear how mechanistically some parameters of beak color could change while one does not. However, some have postulated that a major determinant of beak hue is the conversion efficiency of dietary xanthophyll carotenoids to ketocarotenoids – which gives the reddish color – whereas saturation of the color is dependent on concentration of ketocarotenoids in the tissue (Hill and McGraw, 2006; Koch et al., 2016). The conversion to ketocarotenoids occurs in the mitochondria of the liver and therefore is linked to metabolic efficiency (Powers and Hill, 2021; Powers et al., 2022). Therefore, it is possible that a change in saturation and brightness is a consequence of differences in acquiring or assimilating carotenoids, rather than in differences of the mitochondrial metabolic machinery. In this study we did not show that heat conditioning during development would prevent diminishment of beak color when exposed to a stressor as adults. However, we did demonstrate that even mild environmental conditions can have a persistent effect that is reflected in the beak saturation and brightness of male zebra finches, and potentially underlying physiology as well.

It is in part the function of carotenoids as antioxidants which has led to the suggestions that carotenoid-based ornaments may be reliable signals of condition (Lozano, 1994; Møller et al., 2000). This function has been debated however, as the correlation between circulating carotenoids and oxidative balance has been found to be weak in birds (Costantini and Møller, 2008, Koch et al., 2018). Although their significance with respect to antioxidant function *in vivo* is under scrutiny, it is still thought that carotenoid-based ornaments can act as a good indicator of overall oxidative status. One hypothesis is that these ornaments could be signaling the amount of

other antioxidant molecules available to prevent the oxidization of carotenoids (Hartley and Kennedy, 2004). In support of this "protection hypothesis" studies have found that oxidative stress reduces intensity of coloration, as well as that supplementing non-carotenoid antioxidants results in an increase in carotenoid coloration (Bertrand et al., 2006; Pike et al., 2007; Torres and Velando, 2007; Perez et al., 2008). However, there are also explanations for how carotenoid-based ornaments can be good indicators of oxidative status that are not based on costs/resources, such as the "shared pathway" hypothesis proposed by Hill (2011). In this concept, the pathways that produce ornaments are shared with core cellular processes such as cellular respiration, which is a significant source of ROS production (Hill, 2018). Exposure to stressors can cause dysregulation and inefficiency of mitochondrial respiration (von Schantz et al., 1999). Therefore, individuals more tolerant to stressors by way of prior experiences can better maintain condition, indicated by either more optimal mitochondrial respiration or adaptive synthesis of antioxidant enzymes, both of which should reduce oxidative damage that can negatively impact biological pathways.

One of the aims of our study was to determine to what extent the expression of a carotenoid-based trait signals an individual's underlying oxidative status. Therefore, we examined the relationship between beak color and antioxidant enzyme levels, and how this relationship was altered by developmental conditions and/or adult stress. Our results suggest that the relationship between beak coloration and antioxidant levels depends on prior exposure to a stressor. When looking at the hue of male beaks, we observed an interesting pattern. In males never exposed to heat at either time-point (cont:cont), there was a positive relationship between beak hue and SOD-1 levels in the testes. Therefore, in this group, individuals with higher testes SOD-1 levels tended to have less red beaks after the end of the adult treatment period. Similarly,

the saturation of beak color was strongly negatively correlated with testes SOD-2 in cont:cont males. These patterns could generally be thought to fall under the resource trade-off hypothesis, where individuals must forgo resource allocation to maintenance of the ornament in favor of other physiological processes. In contrast, when looking at the males that experienced the mild conditioning as juveniles, the high heat stressor as adults, or both, a different story unfolds. In these groups there was little correlation between hue and teste SOD-1 levels. Moreso, when looking at the relationship between beak saturation and testes SOD-2 in these heat-exposed groups, we saw that mild:high males exhibited a strong positive relationship between the two variables, in contrast to the negative relationship exhibited by cont:cont males. When examining beak brightness, we saw a similar relationship with regards teste SOD-2 levels due to heat exposure. In the males exposed to both the mild heat conditioning as juveniles and the high heat stressor as adults (mild:high), we saw a strong positive relationship between beak brightness and teste SOD-2 levels, which was significantly different than all other groups. Here, it is difficult to determine what hypothesis best describes these patterns, as to our knowledge there is no data indicating female preference with regards to beak brightness specifically in zebra finches. While it may be commonly assumed that a higher brightness would be preferred, within our experiment we found that lower brightness (darker) correlated with redder beaks in males. These results demonstrate the issue facing researchers when determining the relationship between oxidative status and signal honesty. Whether an ornament can be a reliable indicator of antioxidant levels and/or oxidative damage may depend in large part on their developmental history, and what physiological changes were incurred through plasticity.

While here we show some patterns consistent with the costly signaling/resource trade-offs hypothesis, it is interesting how the relationships often show a decoupling or reversal in

groups exposed to the heat conditioning as juveniles. This decoupling – or loss of strong correlation between variables - potentially suggests that other physiological mechanisms play a significant role in regulating beak color. In this study we showed that heat conditioning as juveniles resulted in altered antioxidant enzyme levels in adulthood. It is probable that other physiological mechanisms of the stress response were altered as well, such as circulating hormones like corticosterone (CORT). Many studies have demonstrated developmental plasticity in the CORT response, that conditions experienced during development can permanently alter baseline and/or stress-induced levels of circulating CORT (Love and Williams, 2008; Banerjee et al., 2012; Schmidt et al., 2014). Elevated CORT levels are generally thought to diminish ornament color, however in zebra finches some studies have demonstrated that increased CORT can correlate with redder beaks (McGraw et al., 2011). It is possible that the beak color response may be similar to CORT's role on the immune response, which can be stimulatory after acute elevations but detrimental when elevated chronically (Sapolsky et al., 2000). In our study, it is possible that there were confounding effects of CORT and antioxidant enzyme levels on beak color, warranting further investigation into the relationship between these two parameters.

In this study, we demonstrated that a mild prolonged stressor during development can act in a conditioning hormetic manner, preventing an increase in oxidative damage in the liver when exposed to a high heat stressor as an adult. It is important, however, that this is not seen as a blanket statement on the comprehensive oxidative status, as we did not see the same pattern indicative of conditioning hormesis in the testes. We also demonstrated that this heat conditioning results in a persistent and seemingly adaptive altered antioxidant enzyme status, once again demonstrating the plasticity of the oxidative defense system in vertebrates and its importance in stress physiology. As illustrated here, the relationship between ornament

expression and underlying physiology is complicated, with hurdles still ahead to disentangle exactly what “condition” they can reliably signal. Whatever physiological adjustments the juvenile mild heat conditioning may have resulted in, it did not prevent diminishment of beak saturation which seemingly did not correlate with resistance to oxidative stress in the tissues measured. As such, it is likely that other physiological responses besides antioxidant enzymes play a large role in mediating expression of these carotenoid-based traits. Future studies would do well to test these ideas with a more comprehensive and temporally variable sampling. Such broader stroke approaches may capture differences in beak color, antioxidant, and damage that sampling such as ours may have missed, and paint a clearer picture of these complicated relationships.

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

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All data are available in the main text or supplementary materials.

Competing Interests

The authors declare no competing interests.

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Chapter 4: Early life thermal conditioning alters heat-shock protein expression in response to an adult thermal stressor

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Summary statement: Male zebra finches exposed to a mild heat conditioning as juveniles had tissue-dependent alterations heat-shock protein expression in response to a high heat stressor in adulthood.

Abstract

Developmental environmental stressors can have instructive effects on an organism's phenotype. This developmental plasticity can prepare organisms for potentially stressful future environments, circumventing detrimental effects on fitness. However, the physiological mechanisms underlying such adaptive plasticity is understudied, especially in vertebrates. We hypothesized that captive male zebra finches (*Taeniopygia castanotis)* exposed to a mild heat conditioning during development would acquire a persisting thermotolerance, and exhibit increased heat-shock protein (HSP) levels associated with a decrease in oxidative damage when exposed to a high intensity stressor in adulthood. To test this, we exposed male finches to a prolonged mild heat conditioning (38 °C) or control (22 °C) treatment as juveniles. Then in a 2x2 factorial manner, these finches were exposed to a high heat stressor (42 °C) or control (22 °C) treatment as adults. Following the adult treatment, we collected testes and liver tissue and measured HSP70, HSP90, and HSP60 levels. We saw varying patterns of HSP expression across tissues. In the testes, finches exhibited lower levels of HSP90 and HSP60 when exposed to the high heat stressor in adulthood if they were exposed to the mild heat conditioning as juveniles. In the liver, finches exposed to the high heat stressor in adulthood had reduced HSP90 and HSP60 levels, regardless of whether they were conditioned as juveniles. While HSP levels were not significantly associated with the reduction in oxidative damage, our results indicate that a mild conditioning during development can have long-lasting effects on HSP expression in response to thermal stressors.

Introduction

During development, environmental conditions have the potential to induce potent and persisting effects on an organism's phenotype. Poor environmental conditions during the developmental period have generally been seen as unfavorable, due to the negative impacts on traits such as immune function, growth, and longevity (Chin et al., 2013; Crino et al., 2014 ; Monaghan et al., 2012). However, under some circumstances stressful environmental conditions may be instructive, resulting in a phenotypic adjustment that is adaptive in poor conditions (Gluckman et al., 2005). Such adaptive phenotypic adjustments often include a persisting change in physiology. Developmental stressors have been demonstrated to have long-lasting effects on physiological systems involved in the stress response specifically, such as steroid hormone production and antioxidant defense mechanisms (Spencer et al, 2009; Noguera et al., 2015; Costantini, 2014).

Another mechanism underlying responses to stressors is the production of heat-shock proteins (HSPs). At the cellular level, protein intermediates that are susceptible to aggregation can become toxic at high quantities (Hartl et al., 2011). To prevent such aggregation, organisms upregulate expression of proteins that act mainly as molecular chaperones to maintain proper proteostasis called heat-shock proteins (Feder and Hofmann, 1999). These proteins exhibit cytoprotective effects by preventing protein aggregates, facilitating protein folding into proper conformation, and regulating clearing of damaged cells (Kalmar and Greensmith, 2009). The upregulation of these proteins is known as the heat-shock response, a mechanism allowing organisms to cope with an array of environmental challenges such as increased temperatures, energy depletion, high concentrations of toxic substances, and oxidative stress (Feder and Hofmann, 1999). Heat-shock proteins are a highly conserved group of proteins, and are not only

synthesized in response to perturbations, but are also constitutively expressed under normal conditions indicating an invaluable role in proper cellular function. There are several families of HSPs grouped according to their molecular weights, with the most well studied and abundant HSPs in the cell being HSP70, HSP90, and HSP60 (Becker and Craig, 1994; Kalmar and Greensmith, 2009). While these families may have slightly different roles, these proteins are necessary for proper protein homeostasis and for mounting a protective response when faced with stressful environments.

While HSP expression can be upregulated in cells due to a variety of stressor types, perhaps the most well documented is the increased upregulation due to heat stress (Kregel, 2002). Heat stress has been demonstrated to increase reactive oxygen species and have negative impacts on a variety of traits such as reproduction, cognition, and motor function (Lin et al., 2006; Walsh et al., 2019; Coomes et al., 2019; Racinais et al., 2019). However, heat stress can have a beneficial effect on lifetime fitness, depending on the timing and magnitude of the exposure, as well as the thermal conditions the individual experiences later in life. This is because exposure to heat stressors, especially during development, can induce an adaptive phenotypic change which allows an individual to better cope with heat stress in the future. For example, zebra finches (*Taeniopygia castanotis*) exposed to a mild heat conditioning as juveniles had an increased tolerance against a higher severity heat stressor in adulthood, suffering less oxidative damage than non-conditioned finches (Costantini et al., 2012). Similar responses have been demonstrated *in vitro* with regards to HSP expression in many species, where exposure to a moderate heat stress results in an upregulation of HSPs (Lindquist, 1986) and not only allows the cell to cope with potential acute consequences of heat-shock, but can also provide resistance to future stressors by incurring a lasting thermotolerance (Lindquist, 1986, Richter et al., 2010).

Therefore, one mechanism underlying a persistent thermotolerance at a whole organism level may be altered constitutive or inducible HSP expression as a result of prior experiences. Unfortunately, most research has examined thermotolerance at the cellular level or focuses on invertebrate species. Studies that examine the link between HSP levels and thermotolerance *in vivo* in non-agricultural vertebrate species are limited.

In this study we used captive zebra finches (*Taeniopygia castanotis*) to test the hypothesis that exposure to a mild heat conditioning early in life results in a persisting change in HSP expression. Specifically, we hypothesized that the conditioned finches would exhibit upregulated HSP expression that would be associated with a decrease in cellular damage when exposed to a high intensity thermal stressor in adulthood due to an acquired thermotolerance. To test these hypotheses, we exposed juvenile male zebra finches to a mild prolonged heat conditioning (38 °C) or control temperature (22 °C) every other day for 28 days. Then as adults, in a 2x2 factorial manner we exposed these individuals to either a high heat stressor (42 °C) or control temperature (22 °C) for three consecutive days. Following the end of the adult treatment period, we collected liver and testes tissue and measured levels of HSP70, HSP90, and HSP60. To analyze the relationship between HSP levels and cellular damage we utilized previously collected data from these individuals where we quantified oxidative damage by measuring protein carbonyls (a protein oxidation marker) and 4-hydroxy-2-nonenal (4-HNE) (a lipid oxidation marker) in the testes and liver respectively. We predicted that the finches exposed to the mild heat conditioning as juveniles would have higher levels of HSP90, HSP70, and HSP60 in the liver and testes tissue after experiencing the high heat stressor as adults, relative to non-conditioned birds. Moreso, we predicted that these conditioned finches would exhibit an inverse

association between HSP and oxidative damage levels in these tissues when exposed to a high heat stressor as adults.

Materials and Methods

For a full description of the animal husbandry, housing, and treatment details, see Hoffman et al. (2018). In short, juvenile (42-45 days post-hatch) male zebra finches (n=22) underwent a mild heat conditioning of 38 °C for 3 hours per day in a brooder (TLC-50, Brinsea, Florida), every other day, for 28 days following a protocol established in Costantini et al. (2012). The remaining juvenile finches (n=17) acted as a control group and were exposed to the room temperature (21-23°C) in brooders in the same fashion. The 38 °C treatment can be considered a mild heat stressor because it is just above the upper critical limit of the thermoneutral zone (TNZ) for zebra finches (Wojciechowski et al., 2021; but see also Calder, 1964). Furthermore, it has been demonstrated that this mild heat conditioning protocol does not increase blood plasma oxidative damage (Costantini et al., 2012). As adults (170-173 days post-hatch), half of the males from each juvenile treatment group were exposed to high heat stressor of 42 °C, or control temperature (21-23 °C) for 3 hours/day for 3 consecutive days in brooders using a 2x2 factorial design. The 42 °C treatment was considered to be a high heat stressor as this temperature is notably above the TNZ for zebra finches, and the 3-day exposure protocol used has been shown to result in an increase in blood plasma oxidative damage (Costantini et al., 2012; Wojciechowski et al., 2021). The result was four final treatment groups that represent all possible combinations of juvenile and adult treatment [Control-Control (cont:cont): n = 8, Control-Heat (cont:heat): n = 9, Mild-Control (mild:cont): n = 12, Mild-High (mild:high): n = 10].

Tissue collection and quantification of proteins

Four hours following the final adult treatment, finches were euthanized via isoflourane inhalation per approved protocol. Finches were then dissected, the testes and liver were collected, flash frozen in liquid nitrogen, and stored at -80 °C until the time when samples were prepped for protein quantification. Protein quantification was done using the left lobe of each liver and both testes, and each sample was homogenized and prepped for gel electrophoresis and Western blotting (using a 2µg/µl sample protein concentration) as previously described (Hyatt et al., 2016). The levels of tissue heat-shock protein 90 (Anti-Hsp90 antibody; ab13495, Abcam), heat-shock protein 70 (Anti-Hsp70 antibody; ab79852, Abcam), and heat-shock protein 60 (Anti-Hsp60 antibody; ab46798, Abcam), and the lipid peroxidation marker 4-hydroxy-2-nonenal (4-HNE, GTX17571, GeneTex) were quantified by Western blot as previously described (Finger et al., 2017; Finger et al., 2018, Finger et al., 2021). Protein oxidation levels were determined by measuring protein carbonyls using a commercially available kit (OxyBlot; s7150; EMD Millipore, Billerica, MA, USA) via Western Blot as described by manufacturer's instructions (Yap et al., 2022). Membranes were stained using Ponceau, acting as a loading and transfer control and imaged using a chemiluminescent system (ChemiDocIt Imaging System (UVP, LLC, Upland, CA, USA).

Statistical analyses

All statistical analysis was done in RStudio (RStudio Team, 2020) using the package "nlme" and graphs were created using the "ggplot2" package. Linear mixed-effects models (LMM) were used to examine the effects of juvenile and adult treatments on heat-shock protein levels, and to examine the relationships between protein levels (HSP and oxidative damage parameters). Effects were determined as significant when $P \leq 0.05$. All models included the nest ID as a random effect to account for multiple males originating from the same genetic nest. To

examine the effects of our juvenile and adult treatments on our protein variables, we initially ran "full" models that included the juvenile*adult treatment interaction term as well as predictors that were believed likely to have a large effect size or plausible biological effect on some aspects of the model. Predictors were then removed when $P \geq 0.1$ to construct the final model. Further post-hoc analyses were done using an LMMs with a categorical variable "treatment" representing the four combinations of juvenile and adult treatments as a fixed factor instead of the juvenile*adult treatment interaction term. This "treatment" variable represents the four distinct treatment groups as a result of the 2x2 factorial design, allowing for post-hoc pairwise comparisons. The assumptions of linearity of the data were tested by plotting the fitted values versus residuals, and the assumptions of normality of residuals were tested via histogram plots of the data for linear models. Regression coefficients were used for estimates of mean change in response variables for linear models. 95% confidence intervals (CI) were used to represent estimates of uncertainty which were determined using the "intervals" package and function.

Results

Testes heat-shock proteins

In the testes, we observed a significant effect of treatment on HSP70. We saw that the finches exposed to the mild heat conditioning as juveniles and the control temperature as adults (mild:cont) had HSP70 levels 2.30 AU ([0.262, 4.34], ±95% CI) higher than finches exposed to the control treatment as juveniles and the high heat stressor as adults (cont:high) (Fig. 1A) (LMM: mild:cont – cont:high (t = 2.41 , d.f. = 15, P = 0.0295)). When examining levels of HSP90 in the testes, we observed an interesting pattern of expression in heat conditioned finches. When exposed to the high heat stressor as adults, the finches that were also exposed to the mild heat conditioning as juveniles (mild:high) had HSP90 protein levels 3.16 AU ([-4.72, -1.60],

±95% CI) lower than those exposed to the control treatment as juveniles (cont:high) (Fig. 1B) (LMM: mild:high – cont:high (t = -4.58 , d.f. = 9, P = 0.0013)). This group (cont:high) also had higher levels of HSP90 proteins in the testes compared to the groups not exposed to the high heat stressor as adults (cont:cont, mild:cont) (LMM: cont:cont – cont:high (β = -4.61, t = -5.31, d.f. = 9, P = 0.0005); mild:cont – cont:high (β = -2.57, t = -3.94, d.f. = 9, P = 0.0034)).

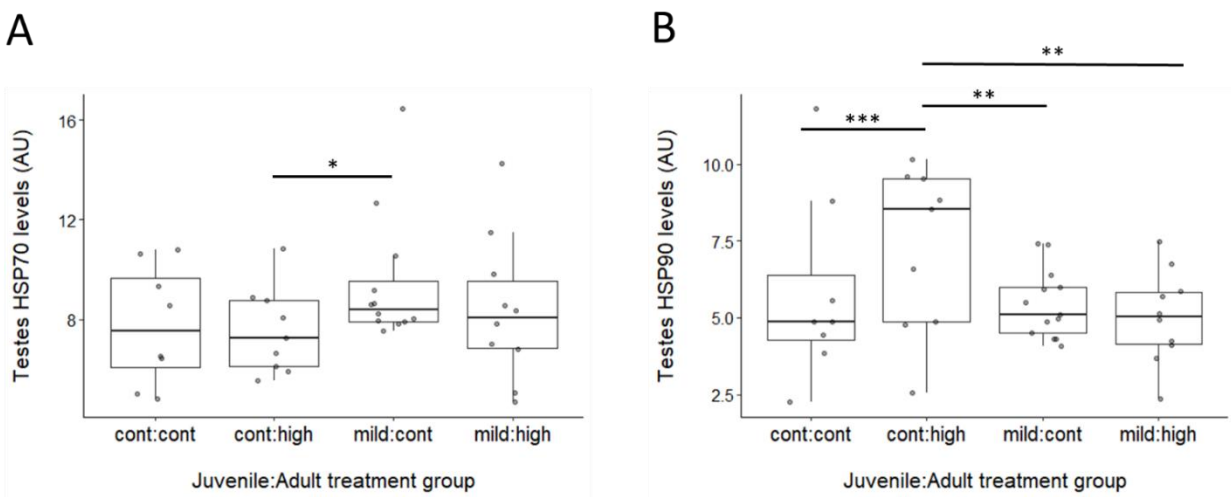

Fig. 1. Effects of juvenile and adult thermal treatments on testes heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90) levels following the adult treatment period (173 DPH). (A) Finches that were exposed to the mild heat conditioning (38 °C) as juveniles but no heat stress as adults (mild:cont) had higher levels of HSP70 in the testes compared to finches that were exposed to the control treatment as juveniles, and then the high heat stressor as adults (cont:high). **(B)** After exposure to the high heat stressor (42 °C) as adults, finches that were exposed to the control temperature treatment (22 °C) as juveniles (cont:high) had higher levels of HSP90 in the testes compared to all other groups. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control or mild heat) and the second word denotes which treatment they were exposed to as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

We saw a similar pattern when looking at HSP60 protein levels in the testes. Once again we observed that when exposed to the high heat stressor as adults, the finches that were also exposed to the mild heat conditioning as juveniles (mild:high) had HSP60 protein levels 0.391 AU ([-0.759, -0.0235] ±95% CI) lower than those exposed to the control treatment as juveniles (cont:high) (Fig. 2) (LMM: mild:high – cont:high (t = -2.30 , d.f. = 13, P = 0.0388)). This group

(cont:high) also had higher levels of HSP60 proteins in the testes compared to the juvenile control group that was also exposed to the control treatment as adults (cont:cont) (LMM: cont:cont – cont:high (β = -0.436, *t* = -2.41, d.f. = 13, *P* = 0.0314) and the conditioned group that was not exposed to the high heat stressor as adults (mild:cont – cont:high (β = -0.205, *t* = -1.35 d.f. = 13, *P* = 0.2006)).

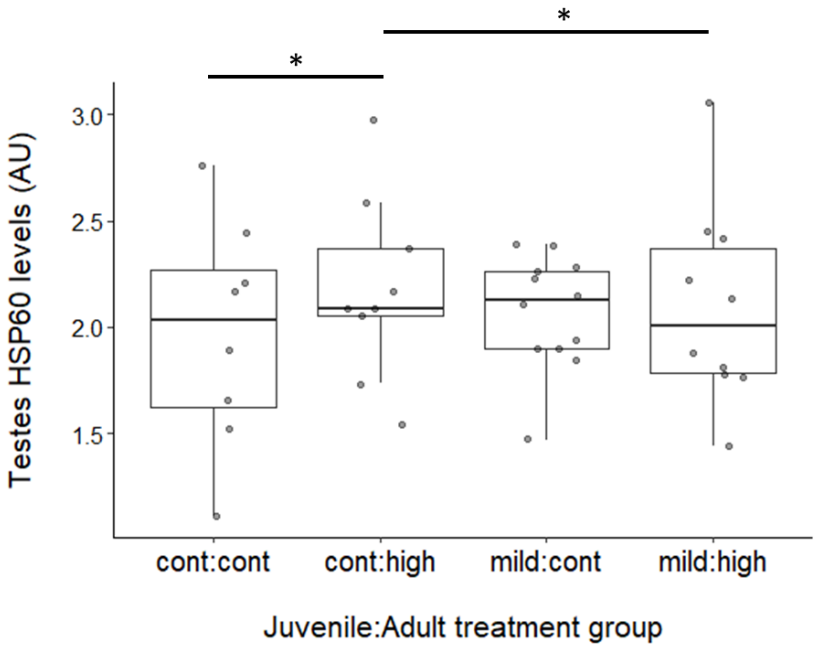

Fig. 2. Effects of juvenile and adult thermal treatments on testes heat-shock protein 60 (HSP60) and protein carbonyl levels following the adult treatment period (173 DPH). (A) After exposure to the high heat stressor (42 °C) as adults, finches that were exposed to the control temperature treatment (22 °C) as juveniles (cont:high) had higher levels of HSP60 in their testes compared to those exposed to the mild heat (38 °C) conditioning (mild:high), and the finches exposed to the control temperature treatment at both timepoints (cont:cont). **(B)** There was no significant effect of juvenile or adult treatment on testes protein carbonyl levels. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control or mild heat) and the second word denotes which treatment they were exposed to as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Liver heat-shock proteins

When examining HSP levels in the liver, we saw remarkably different patterns compared to those in the testes. We observed no significant effect of the juvenile treatment, adult treatment, or their interaction when measuring HSP70 levels in the liver (Fig. 3A) (LMM: Juvenile: β = -

3.01 x 10^{-3}, t = -0.098, d.f. = 14, P = 0.9231; Adult: β = 8.17 x 10^{-4} , t = 0.0235, d.f. = 14, P = 0.98163; J*A: β = -0.0273, t = -0.609, d.f. = 14, P = 0.5524). However, we did see an unexpected effect of treatment on liver HSP90 levels. The finches exposed to the control treatment as both juveniles and adults (cont:cont) had higher levels of HSP90 in the liver compared to finches exposed to the high heat stressor as adults, regardless of the treatment group they were in as juveniles (cont:high, mild:high) (Fig. 3B) (LMM: cont:cont - cont:high (β = -0.161, t = -2.54, d.f. = 13, P = 0.0245); cont:cont – mild:high (β = -0.146, t = -2.27, d.f. = 13, P = 0.0405)).

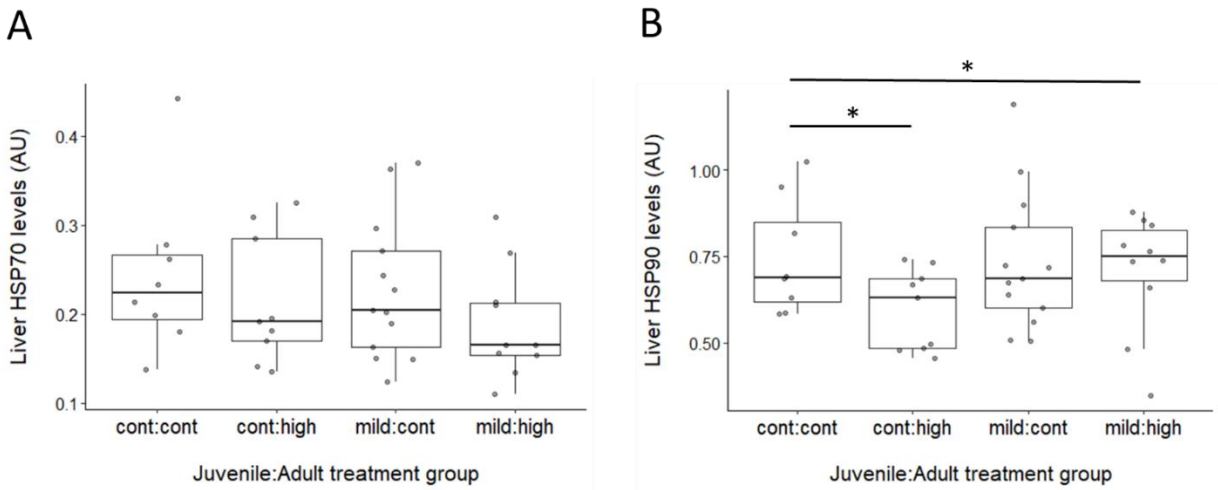

Fig. 3. Effects of juvenile and adult thermal treatments on liver heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90) levels following the adult treatment period (173 DPH). (A) There was no significant effect of juvenile or adult treatment on liver HSP70 levels. **(B)** Finches that were exposed to the control temperature treatment (22 °C) at both the juvenile and adult stage (cont:cont) had higher levels of HSP90 in their liver compared finches exposed to the high heat stressor (42 °C) as adults, regardless of the treatment they received as juveniles (cont:high, mild:high). For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control or mild heat) and the second word denotes which treatment they were exposed to as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

When examining HSP60 levels in the liver, we again saw a pattern contrasting the one seen in the testes. The finches exposed to the control treatment as both juveniles and adults (cont:cont) had higher levels of HSP60 in the liver compared to finches exposed the mild heat conditioning as juveniles (mild:cont), the high heat stressor as adults (cont:high), or both

(mild:high) (Fig. 4) (LMM: cont:cont – cont:high (β = -0.114, *t* = -2.66, d.f. = 16, *P* = 0.0169); cont:cont – mild:cont (β = -0.0997, *t* = -2.51, d.f. = 16, *P* = 0.0230); cont:cont – mild:high (β = -0.0906, *t* = -2.16, d.f. = 16, *P* = 0.0459)).

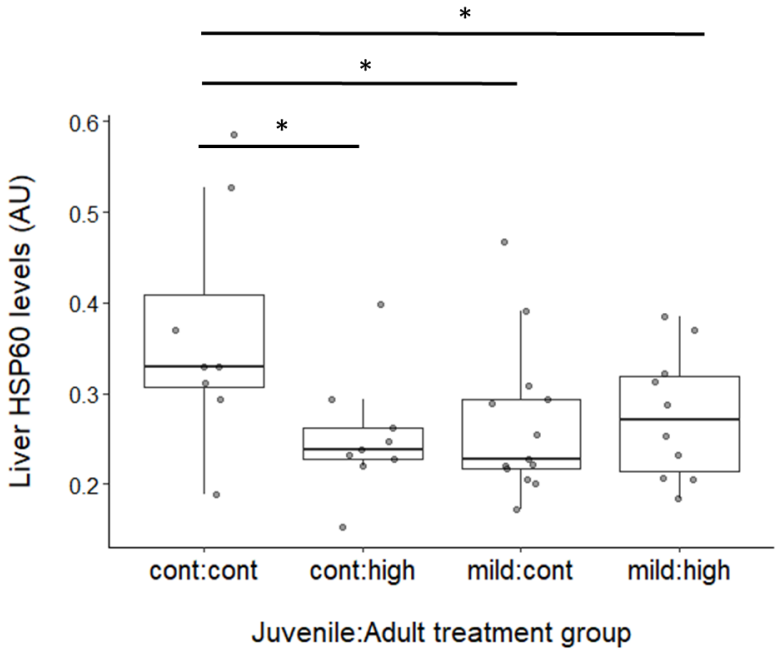

Fig. 4. Effects of juvenile and adult thermal treatments on liver heat-shock protein 60 (HSP60) levels following the adult treatment period (173 DPH). Finches that were exposed to the control temperature treatment (22 °C) at both the juvenile and adult stage (cont:cont) had higher levels of HSP60 in their liver compared to all other groups. For each group on the x-axis, the first word denotes the treatment the finches were exposed to as juveniles (control or mild heat) and the second word denotes which treatment they were exposed to as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Relationships between proteins

Surprisingly, we saw no significant relationships between HSPs and cellular oxidative damage levels, as measured by protein carbonyls (a protein oxidation marker) and 4-hydroxy-2-nonenal (4-HNE, a lipid oxidative marker) (Table S1 and S2). However, we did see some substantial relationships between HSPs types both within and across tissues. When looking at liver tissue, we saw a significant positive relationship between HSP90 and HSP70. For each 1 unit (AU) increase in liver HSP90 levels, we saw a 0.147 AU ([0.0205, 0.273], ±95% CI)

increase in liver HSP70 levels (Table S6) (LMM: t = 2.45, d.f. = 17, P = 0.0253). Interestingly, we saw this same relationship between HSP90 and HSP70 levels when looking across tissues. For each 1 unit (AU) increase in liver HSP90 levels, we saw a 5.18 AU ([1.016, 9.35], ±95% CI) increase in testes HSP70 levels (Table S3) (LMM: t = 2.62, d.f. = 17, P = 0.0178).

Discussion

In this study we set out to determine if exposure to a mild heat conditioning during development conferred a persistent alteration in HSP expression in zebra finches. Moreso, we wanted to determine if the mild heat conditioning induced a lasting thermotolerance, characterized by a negative relationship between HSP expression and oxidative damage when exposed to a high heat stressor later in life. We observed that HSP expression following the high heat stressor in adulthood was in many instances influenced by exposure to the mild heat conditioning during development. However, this pattern varied between HSP types both within and across tissues. When examining HSP70 levels in the testes, we saw that the early life heat exposure was associated with an upregulation of HSP70 in adulthood. Specifically, we saw that the finches exposed to the mild heat conditioning as juveniles and the control treatment as adults (mild:cont) had higher levels of HSP70 in the testes compared to the non-conditioned finches that were exposed to the high heat stressor as adults (cont:high). In some ways, this is not a surprising result. In many instances, thermal preconditioning has been associated with an upregulation of HSP70 expression even after the thermal exposure has ceased. For example, broiler chickens (*Gallus domesticus*) subjected to a heat conditioning protocol of a 1-hour 41 °C exposure daily for a week had an *in vivo* maximization of HSP70 levels, and this upregulation of HSP70 expression persisted for 4 weeks after cessation of the heat conditioning exposure (Wang and Edens, 1998). However, other studies have shown that broiler chickens heat conditioned

during development had lower levels of HSP70 expression following a later acute heat stress, which in one case was associated with a decrease in oxidative damage (Kang and Shim, 2021; Madkour et al., 2021). Therefore, it is interesting that the non-conditioned finches that were exposed to the high heat stressor as adults (cont:high) had reduced HSP70 levels compared to a group not exposed to the high heat stressor (mild:cont). One reason may be due to the point at which the tissue samples were taken post-heat stressor. A previous study on zebra finches reported lower blood constitutive HSP70 (HSC70) levels 4 hours after treatment compared to the pre-stress baseline levels (Finger et al., 2017). Interestingly, this post-treatment trough in HSC70 levels was seen regardless of whether the finches were exposed to the control or 39 °C heat stressor, indicating a potential role of circadian variation or handling stress even in constitutive protein levels. While we did not measure constitutive protein levels in this study, we did also sample tissues approximately 4 hours following the final bout of the 3-day adult treatment period. However, in this study the proteins were measured in the liver rather than the blood as in the previous study, and there is often significant variation in HSP expression between tissues within an individual (Flanagan et al. 1995). In support of this, while we observed differences in testes HSP70 levels, we saw no significant effect of treatment on liver HSP70 levels in this study.

When examining levels of HSP90 and HSP60 in the testes, we saw that the results were contrary to our predictions. We observed that when finches were exposed to the high heat stressor as adults, they had lower levels of HSP90 and HSP60 in the testes if they were previously exposed to the mild heat conditioning as juveniles (mild:high), compared to the non-conditioned finches (cont:high). While it may seem contradictory, such a pattern of reduced HSP levels is often indicative of an acquired thermotolerance (Rajkumar et al. 2015; Vinoth et al.

2015; Rajkumar et al. 2017; Vinoth et al. 2018). For example, broiler chickens exposed to early life thermal conditioning had reduced oxidative stress as well as reduced HSP90, HSP70, HSP60, and corticosterone levels when exposed to a heat stressor at 5 weeks of age (Madkour et al. 2021). When looking at HSP90 and HSP60 levels in the liver, we saw patterns drastically contrasting those seen in the testes. In both cases, the finches exposed to the control treatment as both juveniles and adults (cont:cont) tended to have the highest amounts of HSP90 and HSP60. These (cont:cont) finches had higher levels of HSP90 in the liver compared to finches exposed to the high heat stressor as adults (cont:high, mild:high), regardless of whether they were heat conditioned juveniles. Similarly, the cont:cont finches had higher levels of HSP60 in the liver compared to those exposed to a thermal treatment at either the juvenile or adult stage (mild:cont, cont:high, mild:high). While these differences in HSP expression patterns across tissue type are interesting, similar observations have been seen in HSP research. Variation in tissue HSP expression within an individual in response to stress has been well established in several species, potentially due to certain tissues affording higher levels of protection or having different capabilities for HSP accumulation rate (Flanagan et al., 1995; Woodruff et al., 2022, Lipshutz et al., 2022). For example, in rats (*Rattus norvegicus*) the first organs to accumulate HSP70 following a heat stressor are the liver and small intestine, while the levels of HSP70 in the brain and quadriceps saw no significant increase (Flanagan et al., 1995). However, such differences in HSP70 levels may be due to the temperature differences between tissues, as some organisms such as birds have the physiological capabilities for selective tissue cooling to protect organs such as the brain (Jessen, 2001).

Determining whether HSP levels can act as reliable biomarkers of stress is difficult when there are seemingly conflicting patterns of expression between tissues such as in our study.

On one hand, individuals with high levels of HSPs can be interpreted as having greater levels of protection and are therefore less stressed on a cellular level (Feder and Hofmann, 1999). In such a scenario, individuals with an acquired thermotolerance would have upregulated HSP expression due to prior experience inducing an adaptive phenotype, making them more tolerant to the detrimental effects of heat stress. On the other hand, high levels of HSP expression may indicate a more stressed individual, necessitating a greater stress response. For example, in blue tit (*Parus caeruleus*) nestlings, increased HSP60 levels in the blood were associated with parasitic infection (Arriero et al., 2008). In this scenario, individuals that are more sensitive to heat stress must express more HSPs in comparison to those with a higher degree of thermotolerance. While generally more studies support the former interpretation, our results seemingly lean toward supporting the latter interpretation, at least when looking at HSP levels in the testes tissues. The group that was not conditioned as juveniles and then exposed to the high heat stressor (cont:high) was expected to be the most stressed group at a cellular level, as that same high heat stressor protocol has been demonstrated to increase blood oxidative damage and reduce survival (Costantini et al., 2012; Costantini et al., 2014). Yet, the finches from this group (cont:high) tended to have the highest levels of HSP90 and HSP60 in the testes, including significantly higher levels than the other group which was exposed to the high heat stress as adults, but were also exposed to the mild heat conditioning as juveniles (mild:high).

The idea that these cont:high finches were the most thermally stressed group and the mild:high finches presented an acquired thermotolerance was further supported when looking at damage to at the cellular level. In another study utilizing these same individuals, we observed that when finches were exposed to the high heat stressor as adults, they had lower levels of lipid oxidation (measured by 4-HNE) in the liver if they were previously exposed to the mild heat

conditioning as juveniles (mild:high) compared to the non-conditioned finches (cont:high) (Hoffman et al., *in submission*). However, it is important to note that we saw no differences in oxidative damage in the testes, as treatment had no effect on protein oxidation (measured by protein carbonyls) levels (Hoffman et al., *in submission*). In this study, we observed no significant relationship between HSP levels and these oxidative damage parameters in either tissue. This is surprising, as the upregulation of HSP production has been heavily linked to coping with oxidative insults which can be caused by heat stressors (Feder and Hofmann, 1999; Cheng et al., 2018). One potential reason for these nonsignificant relationships is the previously discussed time-point of our tissue sampling, which was approximately 4 hours following the final bout of the 3-day adult treatment. The amount of oxidative damage incurred following a stressor is influenced by several components of the stress response besides HSP expression, such as antioxidant enzyme production, glucocorticoid secretion, and damage repair mechanisms (Halliwell and Gutteridge, 2015). As such, the difference in response times of these components may obscure the true relationship between an individual part and damage levels, especially when sampling several hours after the end of the stressor exposure. Potentially, if we had sampled immediately after the high heat stressor when HSP would be expected to have been expressed at higher levels, we would have captured a significant relationship. .

Our data did not support the presence of trade-offs between tissues with regards to HSP production, as there was a significant positive relationship between testes HSP70 levels and liver HSP90 levels. The subject of trade-offs and costs with regards to consistently elevated HSP expression is an often underdiscussed component of HSP biology. High concentrations of HSPs inside the cell can have toxic effects and disrupt normal cell functioning potentially through resource costs and their need for translational priority (Feder and Hofmann, 1999; Sørensen et

al., 2003). Such costs of HSP expression often lead to trade-offs with traits such as immune function and reproduction (Sørensen et al., 2008; Sørensen et al., 2010). For example, elevated HSP60 levels in the blood of female pied flycatchers (*Ficedula hypoleuca*) were associated with lower cell-mediated and humoral immune responses (Morales et al., 2006). Although the costs and trade-offs may be minimized if the heat stressor inducing expression is mild (Sørensen et al., 2008; Sørensen et al., 2010). However, whether the trade-offs are beneficial or detrimental to lifetime fitness is dependent upon the context of the environmental conditions (i.e., the environmental matching hypothesis – Monaghan, 2008). As discussed previously, exposure to a heat stressor can induce a phenotypic change that makes an organism more suited to deal with heat stressors later on in life. However, if the organism then has little exposure to heat stressors, then the phenotypic change may be detrimental by incurring trade-offs with potentially no benefit to fitness (Gluckman et al., 2005; Monaghan, 2008). We have seen that the finches exposed to the mild heat conditioning as juveniles had altered HSP levels, as well as reduced oxidative damage in the liver when exposed to the high heat stressor treatment as adults (Hoffman et al., *in submission*). While we did not measure trade-offs in this study, it is likely that the seemingly adaptive alteration in HSP expression as a result of the mild heat conditioning could actually prove detrimental overall if the later thermal environment was not stressful. Supporting this idea, a study done by Costantini et al. (2014) which used the same juvenile conditioning/adult stress protocol saw that when finches were exposed to the mild heat conditioning as juveniles, they had reduced lifespan if they were not also exposed to the high heat stressor treatment as adults (mild:cont vs. mild:high). It is clear that the role of costs and trade-offs, and therefore also potentially life-history evolution, with regards to HSP expression is complex and deserves larger research focus.

Conclusions

In this study, we have shown that a mild heat conditioning during development can result in a persistent alteration in HSP expression in response to a high heat stressor in adulthood. We have also previously demonstrated that this mild heat conditioning results in decreased liver oxidative damage when exposed to the high heat stressor in adulthood, indicating an acquired thermotolerance. However, there was no significant association between HSP and oxidative damage levels contrary to our predictions. We also saw varying patterns of HSP expression between tissues. In the testes, HSP levels seemingly acted as bioindicators of stress, as the non-conditioned finches exposed to the high heat stressor had the highest HSP90 and HSP60 levels. However, heat stress was associated with lower HSP expression in the liver. This variation in HSP levels between tissues highlights the importance of sampling from multiple tissues in order to have a more comprehensive view of an organism's response to a stressor. While it has been well established that adaptive developmental plasticity is often underlined by changes in physiology such as glucocorticoid hormone responsiveness and antioxidant enzyme production, our results demonstrate that a persisting plasticity in HSP expression also potentially plays a role. Future studies would do well to investigate the relationships between HSP expression and levels of other stress response mediators in order to determine if HSPs are part of the underlying mechanisms of adaptive responses to stress and/or if they could act as reliable indicators of an organism's level of cellular stress. Further understanding of physiological responses to heat stress and how those responses are influenced by an organism's prior thermal environment could prove invaluable in predicting how species respond to rapid changes in ambient temperature at a population and species level.

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

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Data accessibility statement

All data used for analyses are available in the main text or supplementary materials and will be available in a digital data repository by manuscript publication.

Conflicting Interests

The authors declare no conflicting interests.

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Supporting Information

	tprotcar			tprotcar			tprotcar			tprotcar			tprotcar			tprotcar		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.87	-2.27 – 4.01	0.568	1.92	-0.66 – 4.50	0.136	2.27	-2.43 – 6.96	0.326	3.20	-1.19 – 7.58	0.144	1.49	-2.22 – 5.19	0.413	1.96	-0.90 – 4.82	0.169
thsp70	0.11	-0.26 – 0.47	0.543															
thsp90				-0.03	-0.44 – 0.39	0.898												
thsp60							-0.24	-2.48 – 2.00	0.825									
lhsp70										-5.35	-18.14 – 7.43	0.388						
testeswt										-5.98	-67.18 – 55.22	0.839						
lhsp90													0.41	-4.80 – 5.62	0.870			
lhsp60																-0.65	-10.24 – 8.93	0.887
Random Effects																		
σ^2	7.097356			7.168180			7.161729			7.211359			7.166141			7.167433		
τ_{00}	0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}		
ICC	0.000000			0.000000			0.000000			0.000000			0.000000			0.000000		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	39			39			39			39			39			39		
Marginal R^2 / Conditional R^2	0.010 / 0.010			0.000 / 0.000			0.001 / 0.001			0.020 / 0.020			0.001 / 0.001			0.001 / 0.001		

Supporting Table S1. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between protein carbonyl levels (AU) in the testes ("tprotcar") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	l4hne			l4hne			l4hne			l4hne			l4hne			l4hne		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.04	0.02 – 0.06	**<0.001**	0.03	0.02 – 0.05	**<0.001**	0.02	-0.01 – 0.04	0.183	0.03	0.02 – 0.04	**<0.001**	0.03	0.01 – 0.05	**0.005**	0.03	0.02 – 0.05	**<0.001**
thsp70	-0.00	-0.00 – 0.00	0.610															
thsp90				0.00	-0.00 – 0.00	0.573												
thsp60							0.01	-0.00 – 0.02	0.111									
lhsp70										0.02	-0.04 – 0.08	0.475						
lhsp90													0.01	-0.02 – 0.04	0.555			
lhsp60																0.02	-0.03 – 0.07	0.526
Random Effects																		
σ^2	0.000202			0.000199			0.000189			0.000198			0.000199			0.000199		
τ_{00}	0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}		
ICC	0.000000			0.000000			0.000000			0.000000			0.000000			0.000000		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	39			40			39			40			40			40		
Marginal R^2 / Conditional R^2	0.007 / 0.007			0.008 / 0.008			0.069 / 0.069			0.013 / 0.013			0.009 / 0.009			0.011 / 0.011		

Supporting Table S2. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between 4-hydroxy-2-nonenal (4-HNE) levels (AU) in the liver ("l4hne") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	thsp70			thsp70			thsp70			thsp70			thsp70		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	8.31	5.94 – 10.68	**<0.001**	5.88	1.54 – 10.22	**0.010**	7.76	5.11 – 10.42	**<0.001**	4.80	1.81 – 7.80	**0.003**	8.80	6.17 – 11.42	**<0.001**
thsp90	0.01	-0.36 – 0.39	0.942												
thsp60				1.20	-0.86 – 3.27	0.235									
lhsp70							2.84	-8.57 – 14.25	0.606						
lhsp90										5.18	1.02 – 9.35	**0.018**			
lhsp60													-1.43	-10.01 – 7.15	0.729
Random Effects															
σ^2	5.067875			4.980229			4.819097			3.736433			5.058897		
τ_{00}	1.419709 $_{nestid}$			1.223224 $_{nestid}$			1.702344 $_{nestid}$			2.040368 $_{nestid}$			1.405742 $_{nestid}$		
ICC	0.218835			0.197184			0.261038						0.217451		
N	21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$		
Observations	39			39			39			39			39		
Marginal R^2 / Conditional R^2	0.000 / 0.219			0.038 / 0.227			0.007 / 0.266			0.182 / NA			0.003 / 0.220		

Supporting Table S3. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP70 levels (AU) in the testes ("thsp70") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	thsp90			thsp90			thsp90			thsp90			thsp90		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	5.48	2.86 – 8.10	**<0.001**	4.55	0.68 – 8.42	**0.024**	6.23	4.00 – 8.45	**<0.001**	6.24	3.18 – 9.29	**<0.001**	5.03	2.70 – 7.36	**<0.001**
thsp70	0.04	-0.26 – 0.35	0.759												
thsp60				0.63	-1.22 – 2.47	0.483									
lhsp70							-1.89	-11.62 – 7.85	0.689						
lhsp90										-0.60	-4.88 – 3.67	0.771			
lhsp60													2.76	-5.05 – 10.56	0.468
Random Effects															
σ^2	4.933630			4.878842			4.856379			4.866339			4.807984		
τ_{00}	0.000000 $_{nestid}$			0.000000 $_{nestid}$			0.000000 $_{nestid}$			0.000000 $_{nestid}$			0.000000 $_{nestid}$		
ICC	0.000000			0.000000			0.000000			0.000000			0.000000		
N	21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$			21 $_{nestid}$		
Observations	39			39			40			40			40		
Marginal R^2 / Conditional R^2	0.003 / 0.003			0.013 / 0.013			0.004 / 0.004			0.002 / 0.002			0.014 / 0.014		

Supporting Table S4. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP90 levels (AU) in the testes ("thsp90") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	thsp60			thsp60			thsp60			thsp60			thsp60		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.80	1.31 – 2.28	**<0.001**	1.94	1.55 – 2.33	**<0.001**	2.16	1.72 – 2.60	**<0.001**	2.08	1.52 – 2.64	**<0.001**	2.20	1.77 – 2.63	**<0.001**
thsp70	0.03	-0.02 – 0.09	0.215												
thsp90				0.02	-0.04 – 0.09	0.421									
lhsp70							-0.37	-2.25 – 1.52	0.687						
lhsp90										-0.00	-0.78 – 0.78	0.997			
lhsp60													-0.40	-1.82 – 1.01	0.555
Random Effects															
σ^2	0.148304			0.136245			0.136789			0.144868			0.137456		
τ_{00}	0.016823_{nestid}			0.036596_{nestid}			0.038885_{nestid}			0.029416_{nestid}			0.036831_{nestid}		
ICC	0.101881			0.211734			0.221349			0.168782			0.211324		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	39			39			39			39			39		
Marginal R^2 / Conditional R^2	0.042 / 0.140			0.016 / 0.224			0.004 / 0.225			0.000 / 0.169			0.008 / 0.218		

Supporting Table S5. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP60 levels (AU) in the testes ("thsp60") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	lhsp70			lhsp70			lhsp70			lhsp70			lhsp70		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.19	0.10 – 0.29	**0.001**	0.24	0.16 – 0.32	**<0.001**	0.19	0.10 – 0.29	**<0.001**	0.23	0.16 – 0.30	**<0.001**	0.25	0.11 – 0.38	**0.001**
lhsp90	0.15	0.02 – 0.27	**0.025**												
testeswt	-1.92	-3.35 – -0.49	**0.011**												
lhsp60				-0.07	-0.33 – 0.19	0.576									
thsp70							0.00	-0.01 – 0.01	0.559						
thsp90										-0.00	-0.01 – 0.01	0.738			
thsp60													-0.01	-0.08 – 0.05	0.670
Random Effects															
σ^2	0.003484			0.004796			0.004134			0.004888			0.004277		
τ_{00}	0.001296_{nestid}			0.001151_{nestid}			0.001966_{nestid}			0.001074_{nestid}			0.001783_{nestid}		
ICC	0.271103			0.193587			0.322324			0.180083			0.294257		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	40			40			39			40			39		
Marginal R^2 / Conditional R^2	0.228 / 0.437			0.007 / 0.200			0.009 / 0.328			0.003 / 0.182			0.005 / 0.298		

Supporting Table S6. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP70 levels (AU) in the liver ("lhsp70") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	lhsp90			lhsp90			lhsp90			lhsp90			lhsp90		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.55	0.38 – 0.72	**<0.001**	0.58	0.40 – 0.76	**<0.001**	0.51	0.31 – 0.71	**<0.001**	0.72	0.55 – 0.89	**<0.001**	0.70	0.39 – 1.01	**<0.001**
lhsp70	0.68	-0.06 – 1.42	0.068												
lhsp60				0.42	-0.19 – 1.03	0.169									
thsp70							0.02	-0.00 – 0.05	0.053						
thsp90										-0.00	-0.03 – 0.02	0.771			
thsp60													0.00	-0.15 – 0.15	0.998
Random Effects															
σ^2	0.028137			0.029333			0.028423			0.030848			0.031739		
τ_{00}	0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}		
ICC	0.000000			0.000000			0.000000			0.000000			0.000000		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	40			40			39			40			39		
Marginal R^2 / Conditional R^2	0.088 / 0.088			0.050 / 0.050			0.102 / 0.102			0.002 / 0.002			0.000 / 0.000		

Supporting Table S7. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP90 levels (AU) in the liver ("lhsp90") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

	lhsp60			lhsp60			lhsp60			lhsp60			lhsp60		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.31	0.21 – 0.40	**<0.001**	0.20	0.07 – 0.33	**0.004**	0.31	0.19 – 0.42	**<0.001**	0.26	0.17 – 0.35	**<0.001**	0.32	0.15 – 0.49	**0.001**
lhsp70	-0.10	-0.52 – 0.32	0.633												
lhsp90				0.12	-0.06 – 0.30	0.169									
thsp70							-0.00	-0.02 – 0.01	0.682						
thsp90										0.01	-0.01 – 0.02	0.468			
thsp60													-0.02	-0.10 – 0.06	0.643
Random Effects															
σ^2	0.009103			0.008689			0.009337			0.009029			0.009324		
τ_{00}	0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}			0.000000_{nestid}		
ICC	0.000000			0.000000			0.000000			0.000000			0.000000		
N	21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}			21_{nestid}		
Observations	40			40			39			40			39		
Marginal R^2 / Conditional R^2	0.006 / 0.006			0.050 / 0.050			0.005 / 0.005			0.014 / 0.014			0.006 / 0.006		

Supporting Table S8. The statistical output of linear mixed models (*LMMs)* used to analyze the relationship between HSP90 levels (AU) in the liver ("lhsp60") and HSP70, HSP90, and HSP60 levels (AU) in the testes and liver ("thsp70", "thsp90", "thsp60", "lhsp70", "lhsp90", and "lhsp60" respectively).

Chapter 5: Developmental thermal conditioning alters the adult glucocorticoid response and DNA damage while mediating variation in lifespan in females

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Keywords: adaptive developmental plasticity, HPA-axis, heat stress, DNA damage, zebra finch, corticosterone, oxidative damage, longevity

Summary statement: Female zebra finches exposed to a mild heat conditioning as juveniles had higher baseline DNA damage and body masses as adults, as well as lower corticosterone levels following a high heat stressor compared to control females. The conditioning elicited a cost of reduced survival in females when they did not also experience the high heat stressor as adults.

Abbreviations:

CORT – corticosterone

HPA-axis – hypothalamic-pituitary-adrenal axis

ELISA – enzyme-linked immunosorbent assay

RADD – Repair Assisted Damage Detection

DPH – days post-hatch

ROS – reactive oxygen species

MR & GR – mineralocorticoid and glucocorticoid receptors

Abstract

Developmental stressors may result in adjustments in physiology that are adaptive, increasing fitness later in life in otherwise damaging environments. Environmental conditions during development can permanently alter responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis which may allow organisms to avoid negative outcomes in future stressful conditions. We hypothesized that zebra finches (*Taeniopygia castanotis*) thermally conditioned early in life will have persistently lower baseline corticosterone (CORT) and DNA damage levels, but higher stress-induced CORT and lower DNA damage compared to juvenile controls in response to a thermal stressor in adulthood. To test this, we exposed juvenile male and female zebra finches to a mild heat stressor (38 °C) or control (22 °C) temperature every other day for 28 days. As adults, these finches were then subjected to either a high heat stressor (42 °C) or control (22 °C) temperature for 3 consecutive days. Later, female finches were also subjected to a standardized capture/restraint stressor protocol. We measured plasma CORT levels and erythrocyte DNA damage prior to and at the end of each treatment period. Female finches conditioned as juveniles had higher baseline DNA damage levels, as well as lower CORT levels 4 hours following the adult treatment. Conditioned finches also had persistently higher adult body masses than juvenile controls, but females had reduced survival if they were not also exposed to the high heat treatment as adults. Our results indicate that developmental plasticity can potentially act adaptively when the early and later life environments match, but detrimental when there is a mismatch.

Introduction

When organisms are confronted with unpredictable environmental stimuli, the result is a host of physiological, behavioral, and molecular changes known as the stress response (Wingfield et al., 1998; Sapolsky et al., 2000). While the stress response promotes the survival of organisms and their return to homeostasis, chronic stressors have been classically seen as negative due to their suppressive effects on traits such as reproduction and growth (Harris & Seckl, 2011; Monaghan et al., 2012; Spencer, Wimpenny et al., 2005). Such stressors occurring during development are of particular interest, as they can have long-lasting effects on phenotype Wada and Coutts, 2021). However, developmental stressors have more recently been recognized for their potential to induce adaptive phenotypic changes (Monaghan, 2008; Gluckman et al., 2005). Such stressor induced adjustments can prove beneficial by acting instructively, allowing the organism to better cope with stressful conditions later in life (Monaghan, 2008; Gluckman et al., 2005). However, whether these developmental stressors act adaptively are often dose-dependent. For example, when juvenile zebra finches were exposed to a mild heat conditioning (38 °C) as juveniles, they exhibited an increased tolerance to a high heat stressor (42 °C) in adulthood and suffered less plasma oxidative damage compared to juvenile controls (Costantini et al., 2012). However, when the finches were exposed to a high heat conditioning (42 °C) as juveniles, they did not have a tolerance to the high heat stressor in adulthood, and suffered increased plasma oxidative damage relative to the mild heat conditioned juveniles (Costantini et al., 2012). Whether such stressors are beneficial not only depends upon the timing and magnitude of exposure, but also the organism's future environmental conditions. If the adult environment is not similar to the early life environment, then the phenotypic adjustment can be maladaptive

(Monaghan, 2008; Harris and Seckl, 2011). However, we still have a poor understanding of the underlying physiological changes involved in such adaptive developmental plasticity.

A potential mediator of these adaptive developmental adjustments is the endocrine system. Part of the underlying physiology of the stress response is the release of glucocorticoid hormones such as corticosterone (CORT), which is under control of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky, 1992). Glucocorticoids have a wide array of effects on both behavior and physiology at baseline levels and demonstrate both circadian and seasonal variation (Landys et al., 2006). However, when confronted with an unpredictable stressor, the result is a steep increase in glucocorticoid levels which acts to suppress traits such as growth, digestion, and reproduction while also directing resources toward activities that may promote short-term survival and recovery (McEwen and Wingfield, 2003; Wingfield and Romero, 2001). While increases in circulating glucocorticoids is an essential part of normal physiological functioning and life-history, chronic stressors leading to prolonged elevated glucocorticoid levels can have lasting negative impacts on traits such as reproduction and immune function and lead to an accumulation of damage at a cellular level (Sapolsky et al., 2000).

Altered glucocorticoid secretion has been deemed as a potential mediator of adaptive developmental plasticity and particularly stress resilience. This is in part because the HPA axis can be "programmed" via conditions during pre- or post-natal development to exhibit altered responsiveness in many vertebrates including birds (Banerjee et al., 2012; Spencer et al., 2009; Schmidt et al.; 2014). For example, starling (*Sturnus vulgaris*) eggs with experimentally elevated yolk corticosterone had a reduced adrenocortical response to a stressor as fledglings (Love and Williams, 2008). While in the past it was thought that such alterations in glucocorticoid secretion was a maladaptive result of a poor developmental environment, such changes have been

recognized for their potential to act adaptively by allowing organisms to better cope with future stressful conditions (Gluckman et al., 2005; Zimmer et al., 2013; Crino et al., 2015). Whether HPA-axis reprogramming will be beneficial to an organism likely requires that the magnitude of the stressor is enough to induce the phenotypic adjustment, but not too high that it has lasting detrimental impacts that outweigh the benefit of altered HPA-axis responsiveness. However, the relationship between glucocorticoids and fitness has remained somewhat unclear, most likely due to variation as the result of life-history and environmental context of individual organisms, which confers differences in resilience to stressors (Vitousek et al., 2018; Schoenle et al., 2018; Schoenle et al., 2021).

Some evidence suggests that a stress-resilient phenotype can be characterized by a greater CORT response and strong negative feedback, which is regulated by glucocorticoid and mineralocorticoid receptors in the brain (Breuner et al, 2008; Schmidt et al., 2012; Vitousek et al., 2019). For example, free-living female tree swallows (*Tachycineta bicolor*) were found less likely to abandon their nest if they had high CORT levels following a stressor and lower CORT levels after injections of dexamethasone, indicating a strong response and negative feedback (Zimmer et al., 2019). Such a phenotype would limit prolonged elevated glucocorticoid exposure while mounting an effective response to deal with the stressor, and has been linked to increased survival levels (Romero and Wikelski, 2010; Patterson et al., 2014). Moreso, these phenotypes with higher CORT reactivity can be induced by exposure to developmental stressors. For example, zebra finches exposed to CORT as nestlings had higher CORT levels in response to a standardized capture/restraint stressor in adulthood (Spencer et al., 2009; Kriengwatana et al., 2014). Historically the focus of the study of glucocorticoids with regards to stress resilience has been on the magnitude of the response, but terminating the response and returning to baseline

levels quickly is most likely also essential for possessing an effective tolerance to stressors. This type of response may attenuate or nullify potential negative effects of stressors such as DNA damage, which has been shown to be caused by acute and chronic stressors in birds (Gormally et al., 2019; Gormally et al.; 2020; Beattie et al., 2024). Damage to DNA can be caused by a variety of stressor types, including heat stress which can also inhibit DNA repair pathways (Kantidze et al., 2016). If such DNA damage is left unrepaired, it can lead to cellular dysfunction, apoptosis, or even carcinogenesis, and can therefore have a significant impact on lifetime fitness (Sedelnikova et al., 2010; Kryston et al., 2011). However, most studies linking glucocorticoid levels and stress tolerance measure only baseline and stress-induced glucocorticoid levels and their relationship with performance outcomes.

In this study we used captive zebra finches (*Taeniopygia castanotis*) to test the hypothesis that exposure to a chronic mild heat stressor during development adaptively modifies the adult HPA-axis response, without incurring cellular damage. Specifically, we hypothesized that the alteration in HPA-axis responsiveness will promote an increased tolerance to stressful conditions in adulthood and avoidance of cellular damage. To test this, we exposed male and female zebra finches to a mild prolonged heat conditioning (38 °C) or control temperature treatment (22 °C) every other day for 28 days as juveniles. Then in a 2x2 factorial manner, we exposed these finches to either a high heat stressor (42 °C) or control temperature treatment (22 °C) for three consecutive days as adults. Later in life, female finches were also exposed to a standardized capture/restraint stressor protocol to determine if the potential HPA-axis responsiveness promotes a cross tolerance to non-thermal stressors. Whole blood was collected before the start and at the end of each treatment period, from which plasma CORT levels were quantified via ELISA and erythrocyte DNA damage was quantified via Repair Assisted Damage Detection

(RADD). We predicted that the finches exposed to the mild heat conditioning as juveniles would exhibit lower baseline CORT levels and unaltered levels of baseline DNA damage later in life. We also predicted that these heat conditioned individuals would exhibit lower CORT and DNA damage levels compared to juvenile controls when measured several hours after exposure to a high heat stressor as adults. Additionally, we predicted that the female heat conditioned finches would exhibit higher CORT levels in comparison to juvenile control females following a standardized capture/restraint stressor.

Materials and Methods

A full account of breeding procedures, general husbandry methods, and thermal treatment procedures can be found in Hoffman et al. (2018) and Hoffman et al. (2023). Briefly, for the juvenile treatment period, a total of 88 zebra finch offspring (47 females and 40 males) were exposed to a prolonged thermal treatment as previously described (Costantini et al., 2012) and utilized in our lab (Hoffman et al., 2018; Hoffman et al., 2023). One group of juveniles (27 females and 24 males) was exposed to a mild heat stressor of 38 °C in a brooder (TLC-50, Brinsea, Florida) for 3 hours per day, every other day for a 28-day period (14 total exposure bouts) beginning at 42-45 days post-hatch (DPH). The other group of juveniles (21 females and 18 males) were exposed to a control temperature treatment of 22 °C in the same fashion. Using a 2x2 factorial design, as adults (170-173 DPH) the finches were exposed to either a high heat stressor (42 °C) or control temperature treatment (22 °C) for 3 hours per day, for 3 consecutive days in brooders as described above. This experimental design resulted in 4 distinct treatment groups that represent all combinations of juvenile and adult treatment [Control-Control (cont:cont): n = 13 females and 8 males; Control - High heat (cont:high): n = 14 females and 9

males; Mild heat - Control (mild:cont): n = 11 females and 13 males; Mild heat - High heat (mild:high): n = 9 females and 10 males]. After the blood sample following the adult treatment was collected, male finches were euthanized and their tissues were collected for use in another study. Females were then kept in individual cages with a male for several months for breeding, after which they were housed in a large flight cage with our general population of female finches. At ~380 DPH, adult finches were exposed to a standardized capture/handling/restraint protocol utilized in avian species to capture baseline and stress-induced plasma CORT levels (Wingfield et al., 1998; Spencer et al., 2009; Zimmer et al., 2019). Three weeks prior to these samples being taken, the experimental females were removed from the general population and placed into individual cages in groups of 2-3 birds in order to acclimate to the change in housing conditions. In this capture/handling/restraint protocol, female finches were captured and handled while baseline blood samples were collected. After this sample was taken, finches were placed in opaque paper bags until 30 minutes after the initial disturbance (entering the room), at which point another blood sample was taken.

Blood sampling

Blood samples were collected from the finches via venipuncture of the brachial vein using a 26-gauge needle. The whole blood was then collected into heparinized microhematocrit tubes which were kept in plastic containers placed on ice. The microhematocrit tubes containing whole blood were centrifuged at 14800 *g* for 10 minutes. The centrifuged samples were then placed back on ice, while the plasma was removed from the microhematocrit tube using a Hamilton syringe and aliquoted into individual 1.5 ml centrifuge tubes. Erythrocytes (RBCs)

were also transferred into 1.5 ml centrifuge tubes using a pipette bulb placed on the microhematocrit tubes. All plasma and RBC samples were then stored at -80 °C until analysis.

In total, blood samples were taken at 6 individual time-points for female finches, and at 4 individual time-points for males. Two blood samples were collected from female and male zebra finches as juveniles. One day before the start of the 28-day juvenile treatment (41-44 DPH) baseline blood samples were taken at ~8:00 local time. For all samples, collection of blood was completed within 3 minutes of the initial disturbance (entering the room). Another blood sample was collected from juvenile finches at the end of the 28-day juvenile treatment period. This post-treatment sample was collected the day following the end of the juvenile treatment (71-74 DPH) at ~8:00 local time in the same manner as the previously described baseline sample. When the birds were adults, another baseline blood sample was taken the day before the start of the adult treatment period (169-172 DPH) as previously described. A post-adult treatment blood sample was also taken on the final day of the treatment. After the final 3-hour treatment bout on the third day, finches were removed from the brooders and placed into cages in pairs. Four hours later, another blood sample was collected as previously described. Later in adulthood (~380 DPH) another baseline blood sample was taken from female finches. A stress-induced sample was then collected 30 minutes after the initial disturbance to capture stress-induced CORT and DNA damage levels.

Corticosterone quantification

The levels of plasma CORT were quantified for all samples using a commercial enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences, no. ADI-901-097). This kit was previously validated and optimized for use with zebra finch plasma and utilized in our lab (Wada

et al., 2009; Rubin et al., 2021). From each sample, 10 µl of plasma was taken and diluted at 1:40 with 1.5% steroid displacement buffer. All samples from each individual finch were included on the same ELISA plate and were randomized as to their position on the plate. The 4 treatment groups (cont:cont, cont:high, mild:cont, mild:high) were also equally represented on each of the 14 total plates run.

DNA extraction from erythrocytes

Genomic DNA was extracted from erythrocytes (which are nucleated in birds) utilizing a commercial kit (NucleoSpin Blood, Macherey-Nagel, no. 740951.250) following the manufacturer's protocol with some modification. We determined that the manufacturer's protocol optimized yield but induced a significant amount of additional DNA damage in the blood (unpublished data) primarily as the resulting of the vortexing steps. Therefore, in our modified protocol, samples were incubated for 30 minutes during the cell lysis stage and vortexing was done at only a single timepoint which occurred following the edition of ethanol after the cells were lysed.

DNA damage quantification

Damage was quantified in the DNA using the Repair Assisted Damage Detection (RADD) assay as previously described (Holton et al., 2018; Lee et al., 2019). The RADD assay measures broad spectrum DNA damage utilizing a cocktail of bacterial repair enzymes. These enzymes excise DNA adducts found throughout genomic DNA, and then the nucleotide biotinylated deoxyuridine triphosphate (dUTP) is inserted at the excision site by DNA

polymerase (Klenow). Cells are then incubated with an anti-biotin-FITC conjugated antibody. The damage amount is then quantified by florescence microscopy.

Statistical analyses

All statistical analysis was done in RStudio (RStudio Team, 2020) using the packages "nlme" and "coxme" and graphs were created using the "ggplot2" and "survminer" packages. Linear mixed-effects models (LMM) were used to examine the effects of juvenile and adult treatments on CORT, DNA damage, and body mass. A mixed effects Cox proportional hazards (Coxme) model was used to examine the effects of juvenile and adult treatments on female survival. Effects were determined as significant when $P \leq 0.05$. All models included the nest ID as a random effect to account for multiple individuals originating from the same genetic nest. To examine the effects of our juvenile and adult treatments on our variables of interest, we initially ran "full" models that included the juvenile*adult treatment interaction term, sex, as well as predictors that were believed likely to have a large effect size or plausible biological effect on some aspects of the model. Predictors were then removed when $P \geq 0.1$ to construct the final model. Further post-hoc analyses were done using an LMMs with a categorical variable "treatment" representing the four combinations of juvenile and adult treatments as a fixed factor instead of the juvenile*adult treatment interaction term. This "treatment" variable allows for post-hoc pairwise comparisons and represents the four distinct treatment groups as a result of the 2x2 factorial design. The assumptions of linearity of the data were tested by plotting the fitted values versus residuals, and the assumptions of normality of residuals were tested via histogram plots of the data for linear models. For Coxme models, assumptions of proportional hazards were examined using a Schoenfeld's test. For linear models, regression coefficients were used for

estimates of mean change in response variables, while hazard ratios were used for estimates in Coxme models. Estimates of uncertainty were represented via 95% confidence intervals, calculated using the functions “intervals” and “confint” for linear regressions and Coxme models respectively.

Results

Corticosterone levels

As expected, we saw no differences in baseline CORT levels between juvenile treatment groups prior to the start of the juvenile treatment ($\beta = 0.280$, $t = 0.804$, d.f. =56, $P = 0.4249$). After the end of the 28-day juvenile treatment period, we saw that the juvenile female and male finches exposed to the mild heat conditioning (38 °C) had plasma CORT levels 1.92 ng/ml ([-0.128, 3.96], ±95% CI) higher than those exposed to the control (22 °C) treatment (Fig.1) (LM: $t = 1.87$, d.f. = 80 $P = 0.0657$). There was no effect of treatment on the net change in CORT levels between the juvenile baseline and juvenile post-treatment samples (LMM: $\beta = 1.67$, $t = 1.50$, d.f. = 52, $P = 0.1403$).

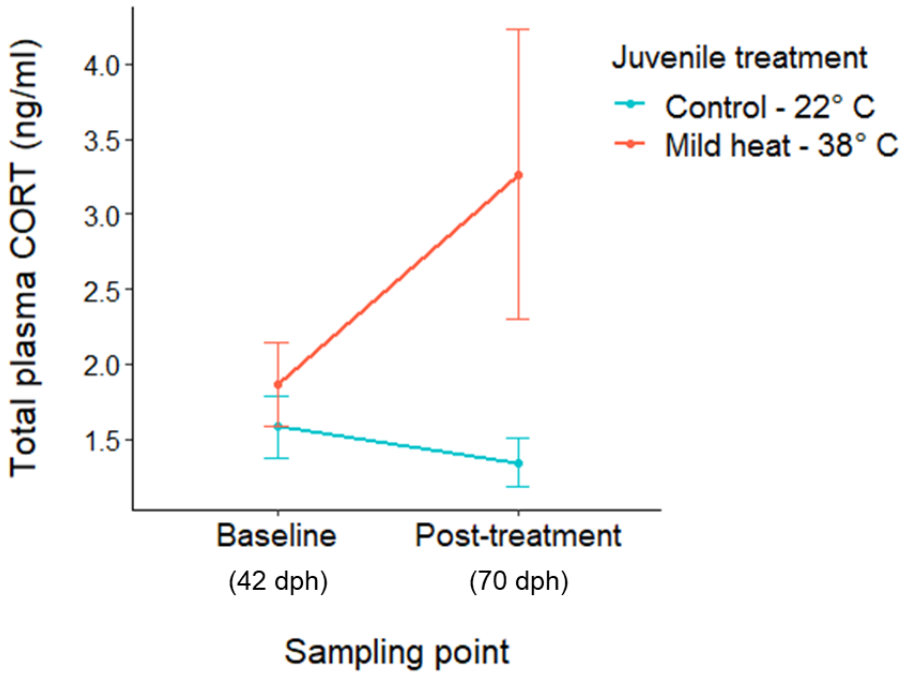

Fig. 1. Total plasma corticosterone (CORT) levels (ng/ml) prior to and after the juvenile treatment period (42-70 DPH). Finches exposed to the mild heat conditioning (38 °C) as juveniles had marginally higher CORT levels when measured at the end of the 28-day treatment period than finches that were exposed to the control temperature treatment (22 °C). Data is represented as the mean ± s.e.m total CORT values at each time point. Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

When examining baseline CORT levels prior to the start of the adult treatment, we saw no differences between juvenile treatment groups (LMM: β = 0.146, t = 0.244, d.f. =56, P = 0.8080). We saw an effect of treatment on CORT levels measured 4 hours after the end of the 3-day adult treatment period, however this effect was only observed in female finches. Female finches exposed to the control (22 °C) treatment as both juveniles and adults (cont:cont) had CORT levels 1.45 ng/ml ([0.394, 2.50], ±95% CI) and 1.27 ng/ml ([0.162, 2.38], ±95% CI) higher than female finches conditioned as juveniles, regardless of the adult treatment they were exposed to (mild:cont and mild:high respectively) (Fig. 2) (LMM: cont:cont – mild:cont (t = 2.89 , d.f. = 18, P = 0.0098), cont:cont – mild:high (t = 2.41 , d.f. = 18, P = 0.0270). We also examined the net change in CORT levels between the baseline and post-adult treatment samples, and once again saw an effect of treatment only in female finches. Among females exposed to the

high heat treatment as adults (cont:high & mild:high), those also exposed to the mild heat conditioning as juveniles (mild:high) exhibited a 2.90 ng/ml ([-4.89, -0.914], ±95% CI) decrease in CORT levels relative to their pre-adult treatment baseline levels, while comparatively those exposed to the juvenile control treatment (cont:high) showed a 0.0963 ng/ml ([-1.53, 1.72], ±95% CI) increase relative to their pre-adult treatment baseline levels (Fig. 2) (LMM: $\beta = -3.00$, $t = -2.46$, d.f. =18, $P = 0.0243$).

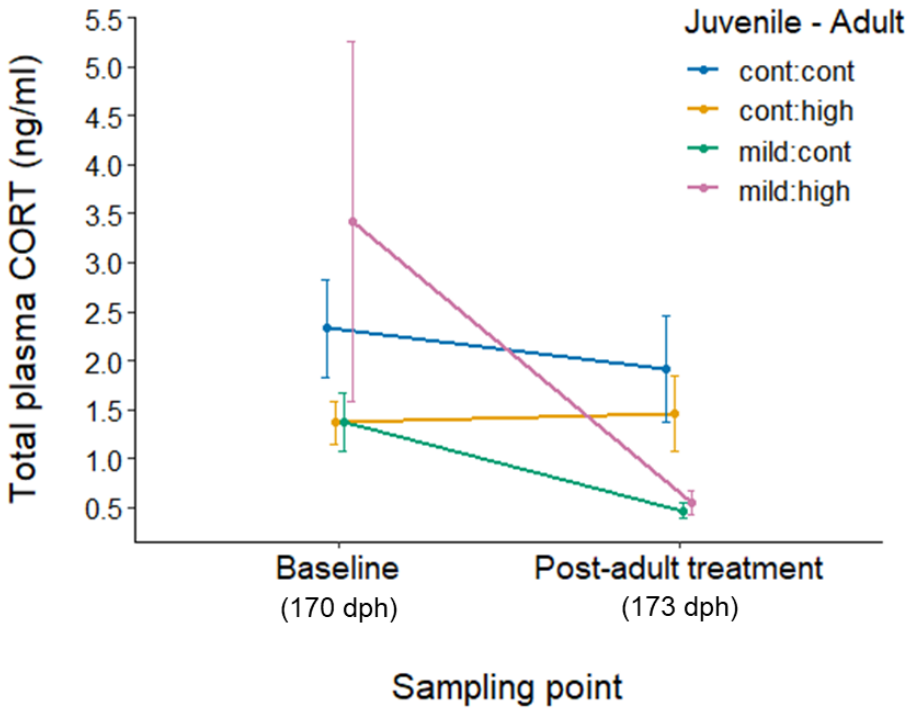

Fig. 2. Total plasma corticosterone (CORT) levels (ng/ml) prior to and after the adult treatment period (170-173 DPH) in female finches. We saw no differences in CORT levels at baseline between juvenile treatment groups prior to the start of the adult treatment period (170 DPH) in females. When measured at the end of the adult treatment period, females exposed to the mild heat conditioning (38 °C) as juveniles had lower CORT levels than females that were exposed to the control temperature treatment (22 °C) as juveniles, irrespective of the adult treatment group. Specifically, mild:cont and mild:high females had lower CORT levels than cont:cont females 4 hours following the end of the adult treatment period. Data is represented as the mean ± s.e.m total CORT values at each time point. Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

We again measured baseline CORT levels in female finches at ~380 DPH and saw no differences between treatments (LMM: Juvenile: $\beta = 0.444$, $t = 0.741$, d.f. = 13, $P = 0.4720$;

Adult: β = 0.587 , *t* = 1.005, d.f. = 13, *P* = 0.3332; J*A: β = -0.0121, *t* = -0.0137, d.f. = 13, *P* = 0.9893). When examining stress-induced CORT levels following the standardized capture/handling/restraint stressor protocol, we also observed no differences between treatment groups (LMM: Juvenile: β = -4.60, *t* = -1.15, d.f. = 14, *P* = 0.2695; Adult: β = -2.47 , *t* = -0.637, d.f. = 14, *P* = 0.5343; J*A: β = 7.15, *t* = 1.19, d.f. = 14, *P* = 0.2557). We also observed no significant effect of treatments when looking at the net change in CORT levels between the baseline and stress-induced samples (LMM: Juvenile: β = -2.61, *t* = -0.957, d.f. = 13, *P* = 0.3562; Adult: β = -0.825 , *t* = -0.312, d.f. = 13, *P* = 0.7603; J*A: β = 4.88, *t* = 1.15, d.f. = 13, *P* = 0.2723).

DNA damage levels

As expected, we saw no differences in baseline DNA damage levels between juvenile treatment groups prior to the start of the treatment (LMM: β = 2.59 $\times 10^{-2}$, *t* = 0.617, d.f. = 31, *P* = 0.5416). We also observed no effect of juvenile treatment on the DNA damage levels measured following the 28-day juvenile treatment period (LMM: β = 9.21 $\times 10^{-3}$, *t* = 0.666, d.f. = 33, *P* = 0.5102) or on the difference in CORT levels between the juvenile baseline and juvenile post-treatment samples (LMM: β = -3.99 $\times 10^{-2}$, *t* = -0.811, d.f. = 18, *P* = 0.4280).

When examining baseline DNA damage levels prior to the start of the adult treatment (169-172 DPH), we saw a significant effect of juvenile treatment, but only in female finches. Adult females exposed to the mild heat conditioning as juveniles had DNA damage levels 2.83 $\times 10^{-2}$ AU ([9.35 $\times 10^{-3}$, 4.72 $\times 10^{-2}$], ±95% CI) higher than those exposed to the control treatment as juveniles (Fig. 3) (LMM: *t* = 3.21, d.f. =14, *P* = 0.0064). However, when looking at DNA damage levels measured 4 hours after the end of the 3-day adult treatment period, we saw no

treatment or sex specific effects (LMM: Juvenile: β = -1.03 x10^{-2}, *t* = -0.592, d.f. = 37, *P* = 0.5571; Adult: β = -2.30 x10^{-2}, *t* = -1.39, d.f. = 37, *P* = 0.1730; J*A: β = 1.70 x10^{-2}, *t* = 0.737, d.f. = 37, *P* = 0.4657; Sex: β = -1.26 x10^{-2}, *t* = -1.008, d.f. = 37, *P* = 0.3199). We also examined the net change in DNA damage levels between the baseline and post-adult treatment samples and again saw no treatment or sex effects (LMM: Juvenile: β = -1.01 x10^{-3}, *t* = -0.0429, d.f. = 26, *P* = 0.9661; Adult: β = -3.07 x10^{-2}, *t* = -1.20, d.f. = 26, *P* = 0.2399; J*A: β = -1.05 x10^{-2}, *t* = -0.287, d.f. = 26, *P* = 0.7766; Sex: β = -1.78 x10^{-2}, *t* = -0.950, d.f. = 26, *P* = 0.3510).

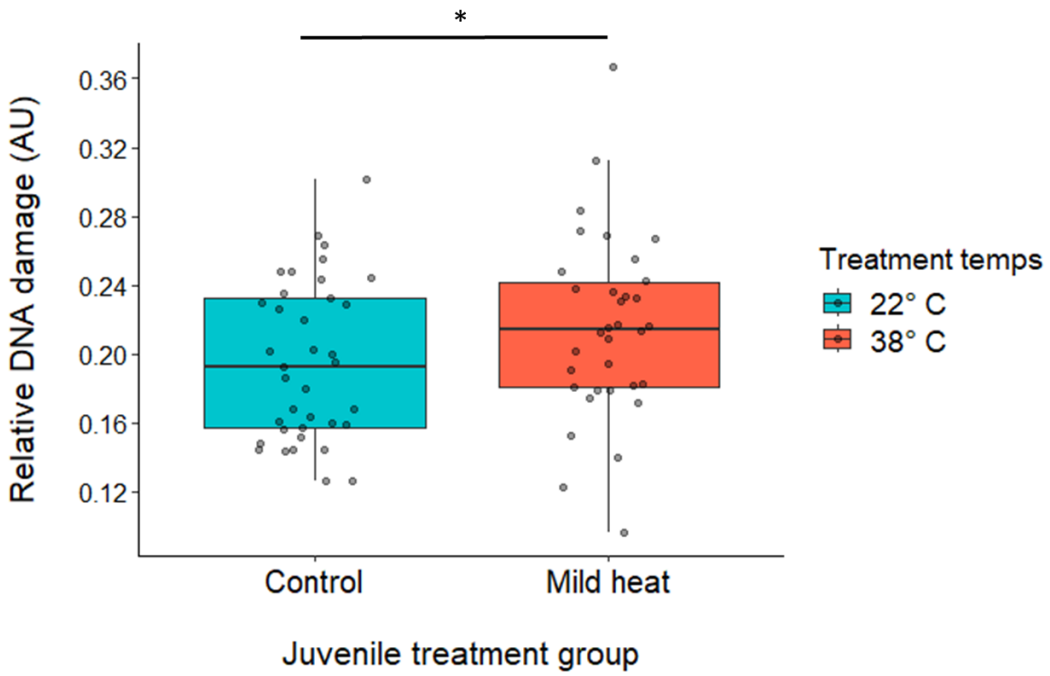

Fig. 3. Total erythrocyte DNA damage levels (AU) prior to the start of adult treatment period (170 DPH) in female finches. Adult females exposed to the mild heat conditioning (38 °C) as juveniles had higher baseline RBC DNA damage levels than those exposed to the control treatment (22 °C) as juveniles before the adult treatment began (170 DPH). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

At ~380 DPH when measuring baseline DNA damage levels in female finches we again saw an effect of treatment. Among the females exposed to the high heat treatment previously as adults (cont:high & mild:high), those also exposed to the mild heat (38 °C) conditioning as

juveniles (mild:high) had baseline DNA damage levels 5.22 x10^{-2} AU ([2.13 x10^{-3}, 0.102], ±95% CI) higher than those exposed to the control treatment as juveniles (cont:high) (Fig. 4) (LMM: t = 2.24, d.f. =14, P = 0.0422). The female finches exposed to the control treatment as juveniles and adults (cont:cont) also had baseline DNA damage levels marginally higher than the non-conditioned and heat stressed finches (cont:high) (Fig. 4) (LMM: β = 4.77 x10^{-2}, t = 2.10, d.f. =14, P = 0.0544). When examining stress-induced DNA damage levels following the standardized capture/handling/restraint stressor protocol, we observed no differences between treatment groups (LMM: Juvenile: β = -2.09 x10^{-2}, t = -1.86, d.f. = 11, P = 0.0900; Adult: β = 1.06 x10^{-2}, t = -1.03, d.f. = 11, P = 0.3265; J*A: β = 2.68 x10^{-2}, t = 1.56, d.f. = 11, P = 0.1476). However, we did see a significant treatment effect when looking at the net change in DNA damage levels between the baseline and stress-induced samples. Among the females exposed to the high heat treatment previously as adults (cont:high & mild:high), those also exposed to the mild heat conditioning as juveniles (mild:high) exhibited a 4.68 x10^{-2} AU ([-9.03 x10^{-2}, -3.25 x10^{-3}], ±95% CI) decrease in DNA damage levels, while comparatively those exposed to the juvenile control treatment (cont:high) showed a 1.64 x10^{-2} ([-2.23 x10^{-2}, 5.51 x10^{-2}], ±95% CI) increase 30 minutes following the standardized capture/handling/restraint stressor (Fig. 4) (LMM: β = -6.32 x10^{-2}, t = -2.54, d.f. = 11, P = 0.0276).

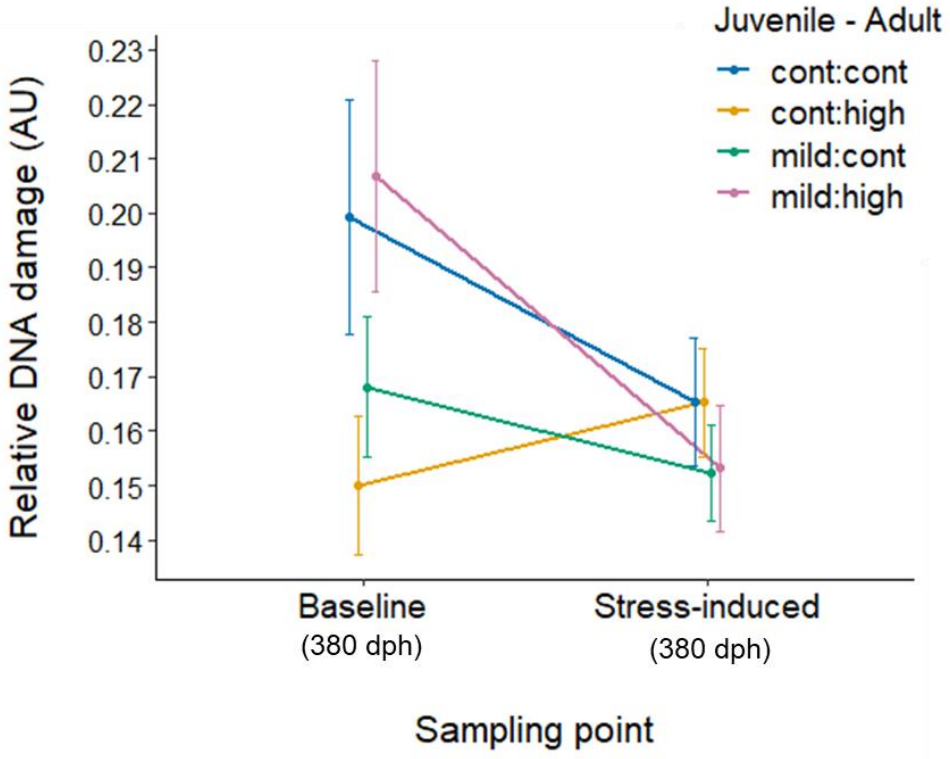

Fig. 4. Baseline and stress-induced erythrocyte DNA damage levels (AU) at ~380 DPH in adult female finches. Females exposed to the control treatment (22 °C) as juveniles and the high heat treatment (42 °C) previously as adults (cont:high) had lower baseline DNA damage levels than females exposed to the mild heat conditioning (38 °C) as juveniles and the high heat treatment (42 °C) previously as adults (mild:high), as well as marginally lower levels than females exposed to the control treatment as juveniles and adults (cont:cont). Data is represented as the mean ± s.e.m total CORT values at each time point. Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Body mass

When measuring body mass prior to the start of the adult treatment we saw no effect of juvenile treatment or sex (LMM: Juvenile: β = 0.140, t = 0.500, d.f. = 55, P = 0.6203; Sex: β = -0.394, t = -1.29, d.f. = 55, P = 0.2031). However, when looking at body mass following the end of the adult treatment, we saw an effect of juvenile treatment but only in female finches. Females exposed to the mild heat conditioning as juveniles had body masses 0.787 g ([0.0905, 1.48], ±95% CI) higher than females exposed to the juvenile control treatment irrespective of the adult treatment they were exposed to (Fig.5) (LMM: Juvenile: β = 0.787, t = 2.36, d.f. = 19, P =

0.0288; Adult: β = 0.220, *t* = 0.678, d.f. = 19, *P* = 0.5062). We also examined the percent change in body mass between the pre- and post-adult treatment measurements and again saw no effects of treatments or sex (LMM: Juvenile: β = 0.244, *t* = 0.171, d.f. = 48, *P* = 0.8650; Adult: β = -1.34, *t* = -0.917, d.f. = 48, *P* = 0.3638; J*A: β = 1.60, *t* = 0.790, d.f. = 48, *P* = 0.4332; Sex: β = 0.122, *t* = 0.118, d.f. = 48, *P* = 9067). This effect of juvenile treatment on body mass seemingly persisted in into adult life, as adult females that were exposed to the mild heat conditioning as juveniles had a 10.35% ([3.15,17.6], ±95% CI) larger increase in body mass between ~170 DPH and ~380 DPH than juvenile control females (Fig. 6) (LMM: *t* = 3.31, d.f. = 8, *P* = 0.0106) and as a result weighed significantly more at ~380 DPH (Fig. 6) (LMM: Juvenile: β = 1.50, *t* = 3.27, d.f. = 7, *P* = 0.0137).

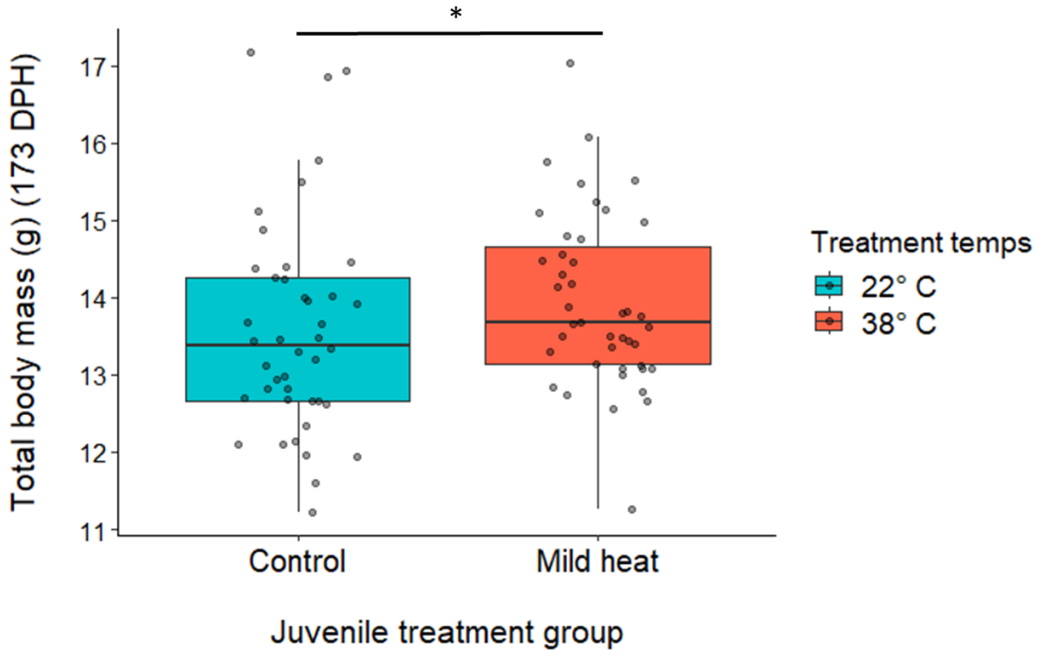

Fig. 5. Total body mass (g) of adult female finches at the end of the adult treatment period (173 DPH). Adult females exposed to the mild heat conditioning (38 °C) as juveniles (mild:cont, mild:high) had higher body mass after the adult treatment period compared to those exposed to the control treatment (22 °C) as juveniles (cont:cont, cont:high), irrespective of the adult treatment group. Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

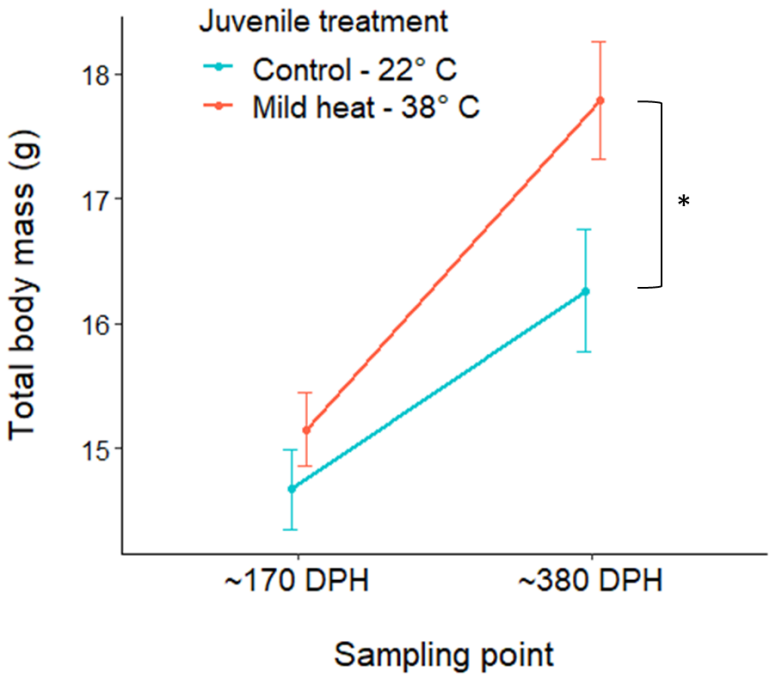

Fig. 6. Baseline body mass (g) levels of female finches prior to the start of adult treatment period (170 DPH) and later in adulthood (~380 DPH). Adult females exhibited no differences in baseline body mass between juvenile treatment groups before the start of the adult treatment (~170 DPH). However, females exposed to the mild heat conditioning (38 °C) as juveniles (mild:cont, mild:high) exhibited a greater increase in mass following the adult treatment period and had higher body masses later as adults (~380 DPH) compared to juvenile control females (cont:cont, cont:high), irrespective of the adult treatment group. Data is represented as the mean ± s.e.m total CORT values at each time point. Significance indicated as follows *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Survival

We saw a significant effect of adult treatment on survival in female finches, however it depended upon the treatment they were exposed to as juveniles. Among females exposed to the mild heat conditioning as juveniles (mild:cont, mild:high), those that were also exposed to the high heat stressor as adults (mild:high) died at a rate 67.8% lower than those exposed to the control treatment as adults (mild:cont) (Fig. 7) (COXME: *Hazard ratio (HR)* = -1.13, 95% CI = [-2.20, -0.0625], $P = 0.038$). The females exposed to the control treatment as juveniles (cont:cont, cont:high) also died at rates 37.8% and 48.9% lower than the mild:cont group respectively (Fig. 7) (COXME: $HR = -0.474$, 95% CI = [-1.47, 0.518], $P = 0.350$; $HR = -0.671$, 95% CI = [-1.64, 0.298], $P = 0.170$).

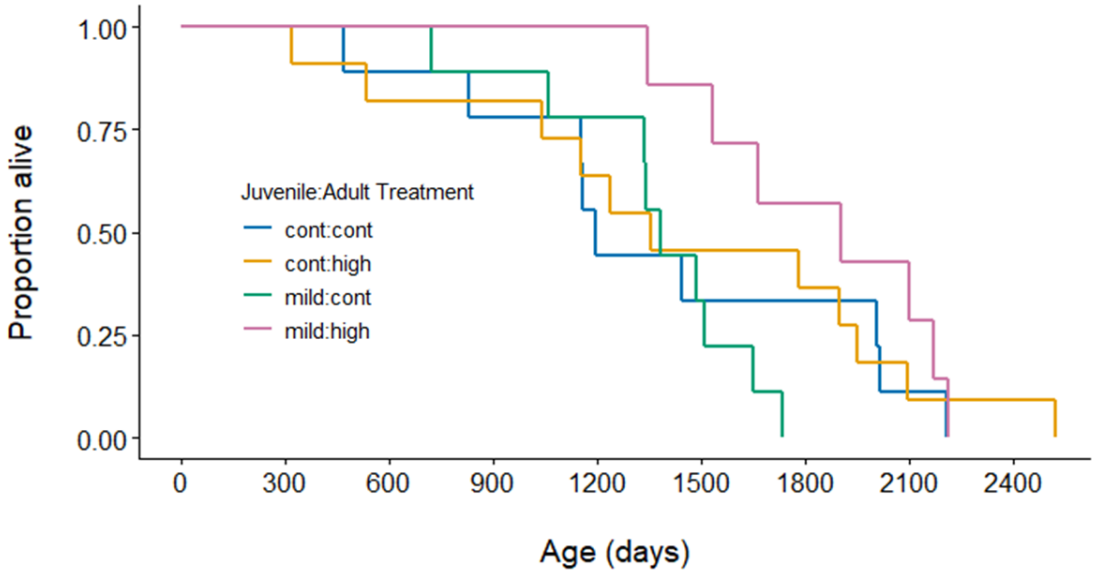

Fig. 7. Survival of female finches and the effect of juvenile and adult treatments. When females were exposed to the mild heat conditioning (38 °C) as juveniles, it came at a cost of reduced survival if they were exposed to the control treatment (22 °C) as adults rather than the high heat treatment (42 °C) (mild:cont vs. mild:high).

Discussion

The goal of our study was to test whether exposure to a prolonged mild heat conditioning during development adaptively modifies the HPA-axis, promoting an increased tolerance to stressful conditions in adulthood. Moreso, we sought to determine whether the alternation in HPA-axis functioning will be characterized by a faster return to baseline CORT levels and reduced DNA damage following exposure to a high heat stressor. While many of the results did not match our predictions, we did observe sex specific effects of treatments on HPA-axis responsiveness, DNA damage, body mass, and survival. When examining the effects of the juvenile treatment immediately following its cessation, we saw no significant effects of the mild heat conditioning on CORT or DNA damage levels. Interestingly, we did see persisting effects of the juvenile treatment on both CORT and DNA damage levels throughout the adult life, but only in female finches. Prior to the start of the adult treatment, we saw no influence of the mild heat

conditioning on baseline CORT levels in males or females. However, contrary to our predictions, female finches exposed to the mild heat conditioning as juveniles had higher baseline DNA damage as adults than those exposed to the control treatment as juveniles. Damage to DNA can result in mutations which are thought to be a significant determinant of ageing and lifespan (Schumacher et al, 2008; Freitas and de Magalhães, 2011). If such damage is left to accumulate without repair, it can be associated with negative life-history impacts in many species, including birds. For example, in wild common yellowthroat (*Geothlypis trichas*) males, DNA damage was correlated with higher overwinter mortality (Freeman-Gallant et al., 2011).While this increase in DNA damage in conditioned finches did not fit our predictions, sufficient DNA repair rates and other anti-damage regulators such increases in baseline DNA damage levels may be ephemeral and not lead to reductions in fitness or trade-offs (Wada, 2019; Zhang and Hood., 2016). For example, the long-lived ecotype of western garter snakes (*Thamnophis elegans*) has RBCs more susceptible to DNA damage, but also has higher repair rates as well as more efficient mitochondria and antioxidant defenses than the short-lived ecotype (Robert and Bronikowski, 2010). While it may seem contradictory, there is also a chance that the heat conditioned finches actually had lower rates of DNA damage, as variation in cellular turnover of RBCs can cause a misrepresentation of the true DNA damage rates. For example, grass snakes (*Natrix natrix*) with higher standard metabolic rates had increased plasma oxidative damage and reduced erythrocyte antioxidant levels (Bury et al., 2020). However, these highly metabolic individuals did not have increased levels of erythrocyte DNA damage when measured due to increased erythrocyte turnover which acts as a method of damage removal (Bury et al., 2020; Vaanholt et al., 2008).

Potentially, the differences in baseline DNA damage between treatment groups could be indirectly due to differences in body mass. Metabolic rates increase with body mass in birds and

many other species (Nagy, 2005; Hudson et al., 2013). One unavoidable byproduct of cellular metabolism is the production of reactive oxygen species (ROS) which cause oxidative damage by reacting with biomolecules such as proteins, lipids, and DNA (Halliwell and Gutteridge, 2015). As such, there is generally a positive association between whole body metabolic rate and oxidative DNA damage (Adelman et al., 1988; Greenberg et al., 2000; Redman et al., 2018). The finches exposed to the mild heat conditioning as juveniles had higher body masses as adults than control juveniles in this study, and therefore potentially higher metabolic rates as well. All though we did not measure metabolic rates, it is possible that the increased baseline DNA damage levels seen in conditioned female finches are the indirect result of larger masses, although the statistical relationship between damage levels and body mass was not significant.

This increase in mass resulting from the mild heat conditioning is somewhat surprising, as it is thought that generally larger body sizes are unfavorable in warm environments because it reduces heat dissipation by decreasing the surface area to mass ratio (Brown and Lee, 1969; McNab, 1980). However, when prolonged increases in ambient temperature are expected, larger body sizes may act beneficially by decreasing water loss. Death due to dehydration rather than hyperthermia may be a larger threat to smaller avian species, as smaller birds lose water at a proportionally higher rate under increasing ambient temperatures (McKenchnie and Wolf, 2010; Allbright et al., 2017). Small songbirds benefit the most from increasing body temperatures with increasing ambient temperatures, as it forms a gradient for passive heat dissipation that limits water lost by evaporative cooling (facultative hyperthermia) (Gerson et al., 2019). However, prolonged increases in body temperature can increase oxidative stress (Lin et al., 2006; Boyles et al., 2011). Zebra finches specifically may prioritize water conservation over avoidance of increases in body temperature, as it has been shown that during exposure to high ambient

temperature, they will increase facultative hyperthermia and limit evaporative water loss (Cooper et al., 2020). In birds, larger body sizes are also associated with increased survival and lifespan, traits that have significant impacts on individual fitness and population dynamics (Furness and Speakman, 2008; Naef-Daenzer and Grüebler, 2016). Therefore, increased body masses may be adaptive even when high ambient temperatures are expected, as long as the potential cost of increased oxidative stress does not outweigh the benefits.

We also examined how exposure to the adult treatment affected CORT and DNA damage levels, and how those parameters were influenced by the juvenile mild heat conditioning. Female finches exposed to the mild heat conditioning as juveniles had lower CORT levels 4 hours after the end of the adult treatment, compared to the juvenile controls, potentially suggesting that they had a faster return to baseline levels. This pattern was seen regardless of whether they were exposed to the high heat stressor (42 °C) or control (22 °C) treatment, potentially indicating a role of handling stress. However, when comparing these post-adult treatment CORT levels to their baseline levels, we saw something interesting. After exposure to the high heat stressor treatment, conditioned females also showed a substantial decrease in CORT relative to their baseline levels, whereas juvenile control females showed a slight increase. Interestingly, this pattern seen in the females exposed to the mild heat conditioning fits a scenario described by a study which sought to model the optimal hormonal response when an organism is exposed to an acute stressor, and how this optimal was influenced by levels of damage repair (Taborsky et al., 2022). The model predicted that organisms with higher damage repair rates would have higher baseline and higher peak hormone levels when encountering an acute stressor, because they will be able to remove the resulting higher levels of damage rather than allowing it to accumulate. The model also predicts that robust repair rates would be associated with a temporary drop in

hormone levels that go below baseline levels, following the strong stress-induced hormone peak (Taborsky et al., 2022, *see Fig. 1B*). This dip in hormones after the stress-induced peak allows for excess damage accrued during the peak levels to be repaired and prevent accumulation before returning to baseline hormone levels. When an increase in stressor frequency and predictability (autocorrelation) is accounted for in the model, the dip in stress hormones becomes larger and the time taken to return to baseline levels becomes longer (Taborsky et al., 2022, *see Fig. S1A*). While we are not aware of any empirical data that supports these patterns, our lab has previously demonstrated the same post-stressor dip in constitutive heat-shock protein 70 levels below baseline following a similar heat stressor (Finger et al., 2017).

The differences in post-treatment CORT levels are likely due to differences in glucocorticoid receptor physiology. As previously discussed, efficient negative feedback is thought to be an integral part of a stress-resilient phenotype as it limits the total exposure time of elevated CORT levels after coping with the initial stressor. Negative feedback of the glucocorticoid response is regulated by glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in the brain, and it has been demonstrated in birds that early-life stressors can permanently affect the number of these receptors and their response to stressors (Zimmer and Spencer, 2014; Banerjee et al., 2012). Therefore, it's possible that the females exposed to the mild heat conditioning had increased GR and/or MR giving them negative feedback that is better attuned to coping with stressor and minimizing negative outcomes, resulting in the dip in CORT levels during the recovery period after the treatment. Another potential explanation for the differences in post-adult treatment CORT levels is an interaction between the treatment and circadian rhythms. Diel rhythms in both baseline and stress-induced CORT levels have been demonstrated in avian species, with CORT levels generally highest during the inactive phase at

night (Breuner et al., 1999; Romero and Remage-Healey, 2000). While we saw no differences in baseline CORT levels between treatment groups, those measures were taken at ~0800 local time at the start of the active phase for the finches, when generally baseline and stress-induced CORT levels are higher than later in the day during the active phase in avian species when our post-adult treatment samples were taken (~1600 local time) (Breuner et al., 1999; Romero and Remage-Healey, 2000; Schwabl et al., 2016). It is possible at 4 hours post-treatment the females were fully "recovered" from a CORT standpoint. Potentially, the females exposed to the mild heat as juveniles may have had lower baseline CORT levels rather than more efficient negative feedback, but only at certain points in the circadian cycle.

Contrary to our predictions, we did not see any differences in DNA damage following the adult treatment. This is surprising, as this high heat stressor protocol has been demonstrated to cause an increase in plasma oxidative damage and lipid oxidative damage in the liver of zebra finches (Costantini et al., 2012; Hoffman et al., 2024 *in revision*). It's possible that the sampling time-point was sufficient to detect differences in damage types in some tissues, but not in RBC DNA damage. Tissues and cell types often differ on the timescale in which defenses such as antioxidant enzymes are mobilized, and how long it takes for damage to incur after exposure to a stressor (Costantini, 2019). There are also differences in the rate of repair due to the type of damage and tissue, as some damage can be fully repaired within hours, while other types may take weeks (McEwen, 2008; Consiglio et al., 2010; Gormally et al., 2019). Therefore, it's possible that DNA damage is repaired at a faster rate than damage to other biomolecules due to its potential to result in somatic mutations and cellular apoptosis, resulting in increased aging rates and shortened lifespans (Evans et al., 2004; Tann et al., 2011; Wolters and Schumacher, 2013).

Later in adulthood (~8 months following the adult treatment period) we again saw that there were no differences in baseline CORT levels between treatment groups, nor were there differences in stress-induced CORT levels. This lack of difference in stress-induced CORT levels further supports the idea that the lower CORT levels previously observed in heat-conditioned female finches 4 hours after the adult treatment was due to an extended below baseline dip in CORT levels, rather than a reduced stress-induced peak. At this time-point, we saw a difference in baseline DNA damage levels between groups once again. Among female finches exposed to the high heat stressor during the adult treatment period, those that were also exposed to the mild heat conditioning as juveniles (mild:high) had higher levels of DNA damage than the juvenile controls (cont:high). They also showed a difference in how DNA damage levels responded to stress, as the mild:high females showed a decrease in DNA damage 30 minutes after the stressor exposure relative to their baseline levels, whereas the cont:high females saw a slight increase. Interestingly, this means the two groups had no differences in their stress-induced damage levels, nor was there any differences between any of the 4 treatment groups (cont:cont, cont:high, mild:cont, mild:high). This result could be another indication that the mild:high females had increased repair rates relative to the cont:high females. It may be considered difficult to classify this phenotype of mild:high females as adaptive, as they still had higher baseline and similar stress-induced DNA damage levels. Again, such damage may not always lead to cellular dysfunction and negative impacts on life-history traits if anti-damage regulators are sufficient (Wada and Heidinger, 2019). For example, some longer lived species exhibit relatively high amounts of oxidative damage, and human centenarians exhibit higher levels of protein oxidative damage than the average human (Andziak et al., 2006; Brys et al., 2007; Nagase, et al. 2018).

DNA damage is thought to be a major contributor to the rate of aging and a mediator of life-history trade-offs (Freitas and De Magalhães, 2011). Moreso, glucocorticoid hormones have been found to decrease DNA repair rates and act as mediators of oxidative stress (O'Brian et al., 1993; Flint et al., 2007; Costantini et al., 2011; Angelier et al., 2018). Therefore, we examined how treatment influenced the survival of female finches in our study. We saw that among those exposed to the mild heat conditioning as juveniles, they had lower survival rates if they were not exposed to the high heat stressor as adults (mild:cont). Interestingly, we did not see reduced rates of survival in the juvenile controls exposed to the high heat stressor as adults (cont:high) as previously reported using this protocol (Costantini et al., 2014). In line with the "environmental matching hypothesis" (Monaghan, 2008), the phenotypic adjustment resulting from the early life mild heat conditioning was maladaptive when the females did not encounter a "matching" environment via exposure to a heat stressor later in life. This cost of the phenotypic plasticity was likely mediated in large part by DNA repair capabilities and/or the production of ROS, which are often considered good indicators of biological age and predictors of lifespan (Lambert et al. 2007; Buffenstein et al. 2008; Whitehead and Grigliatti, 1993; Hyun et al., 2008). A study done by Costantini et al. (2012) using the same mild heat conditioning protocol in zebra finches demonstrated that among female zebra finches exposed to the mild heat conditioning as juveniles, they showed a decrease in RBC thiol antioxidants across the adult treatment period if they were exposed to the control treatment (mild:cont) rather than the heat stressor (mild:high) (Costantini et al., 2014). As pointed out by the authors, such a decrease in thiols could make these individuals more susceptible to oxidative damage when conditions are seemingly benign. It is believed that such an accumulation of oxidative damage leads to loss of integrity and degradation of cells and tissues over time, resulting in aging and culminating in death (Harman,

1981; Finkel and Holbrook, 2000). It is surprising then that the mild:cont females did not have higher DNA damage levels than mild:high females while also having reduced survival. This perhaps points to the influence of other underlying mechanisms such as developmental plasticity in mitochondrial biology, which can result from exposure to stressors to meet energetic demands that can result in long lasting changes in aerobic cellular metabolism and mediate the life-history of individuals (Hood et al., 2018; Pichaud et al., 2019; Gyllenhammer et al., 2020; Cossin-Sevrin et al., 2022; Stier et al., 2022).

Another somewhat contradictory explanation for the reduction in longevity exhibited by mild:cont females in our study, is that conditioned females had ROS levels below the optimal amount under benign conditions which are inhibiting cellular signaling pathways. At moderate concentrations, ROS can act beneficially by initiating cellular signaling responses for essential processes such as stem-cell differentiation and renewal (Schieber and Chandel, 2014; Pomatto and Davies, 2018). Furthermore, low levels of ROS have been shown to stimulate cell proliferation and activate cellular stress pathways to upregulate production of cellular protective enzymes both *in vitro* and in *vivo*, which can attenuate tissue deterioration and increase longevity via stress tolerance (Grune et al., 1997; Sitte et al., 2000; Pickering et al., 2012; Pickering et al., 2013; Ristow and Schmeisser, 2011; Raynes et al., 2016; Pomatto et al., 2017). While ROS are not always detrimental, antioxidants are also not always beneficial. When levels of antioxidants are too high relative to the amount of ROS, the ROS mediated signaling pathways can be disrupted (Sies, 2014; Ristow and Schmeisser, 2011).

It is possible that in this study the mild heat conditioning resulted in a persistent upregulation of cellular defense enzymes (e.g. antioxidants) that are adaptive under highly stressful conditions when ROS increase to potentially damaging levels. However, such a change

could be maladaptive in benign conditions where stressors are not encountered and basal ROS levels are low, by reducing them below the optimal levels to which they were adapted to. Furthermore, it has been demonstrated in birds specifically that stressors experienced during development can result in variation in antioxidant enzyme production as adults, potentially in a manner akin to HPA-axis programming (Marasco et al., 2013; Costantini, 2014; Zimmer and Spencer, 2015; Noguera et al., 2015). Therefore, the mild:cont females may have ROS levels too low for proper cellular signaling, resulting in a loss of cellular homeostasis and reduced survival. This idea that conditioned finches had a persistent upregulation in cellular defenses is partly supported by another study using these birds, which showed that mild:cont males had higher liver SOD2 and testes GPx antioxidant enzymes in adulthood compared to non-conditioned males (cont:cont) (Hoffman et al., *in revision*). However, low levels of ROS could also be due to a decreased production of basal ROS, rather than an increased production of antioxidant enzymes. This can occur through reduced mitochondrial efficiency, which is the level of coupling of ATP synthesis with oxygen consumption during oxidative phosphorylation (Ricquier, 2007; Divakaruni and Brand, 2011). Such uncoupling can reduce ROS production and demonstrates some degree of developmental plasticity in birds (Ricquier, 2007; Divakaruni and Brand, 2011; Helle et al., 2012; Salin et al, 2015; Galli et al., 2016; Casagrande et al., 2020).

In this study we saw pronounced sex specific effects of our thermal treatments on HPA-axis responsiveness and DNA damage. Differences in phenotypic attributes between sexes are not uncommon, including sensitivity to stressors (Lande, 1980; Bangasser and Wicks, 2017). Males and females go through divergent early life developmental trajectories that have different physiological requirements which may influence how they respond to stressors during this time (Badyaev, 2002). This may result in a higher potential for developmental stressors to influence

phenotype to a larger degree in one sex, leading to larger variation in adulthood. This is often seen in birds, where conditions during development effect adult survival and performance differently between males and females (Tschirren et al., 2003; Velando, 2002; Kalmback et al., 2005). Differences between sexes in their response to stressors as adults has been well documented, and while it depends on the timing of the stress and the trait being measured, in some situations females have been found to be more stress-sensitive (Bale and Epperson, 2015; Bangasser and Wicks, 2016; Hodes and Epperson, 2019). Many studies including this one have seen sex-specific variation in important life-history traits such as DNA damage and longevity, mediated by variation in stress response mechanisms (Huber et al., 2019; Tower et al., 2020). Sex-specific variation in HPA-axis responsiveness is key in mediating such effects, likely in part because testosterone and estrogens can influence the HPA-axis and therefore sensitivity to stressors (Patchev and Almeida, 1998; Kajantie, 2006; Small and Schoech, 2015). While we did not measure the survival of males in this study, it would have been interesting to see if males would have exhibited a similar pattern as females given the female-only effects of treatment on DNA damage and CORT levels, especially given that zebra finches differ in thermoregulatory capacities between sexes (Pessato et al., 2022; Pessato et al., 2023).

Conclusions

In this study we demonstrate that such mismatches between underlying physiology and benign environments can occur within a single generation via developmental plasticity. However, we see that such plasticity can be adaptive when the later life environment "matched" and finches were again exposed to heat stress (mild:high). Females exposed to the mild heat conditioning as juveniles had higher baseline DNA damage levels, as well as lower CORT levels

4 hours following the adult treatment. Alone, these effects are hard to classify as adaptive or even neutral, however, the benefit of the phenotype was clearly seen when looking at other fitness-related traits. Conditioned females also had persistently higher body masses than juvenile controls, and if they were also exposed to the high heat treatment as adults (mild:high), still maintained survival rates similar to cont:cont females. These traits often have strong links to life-time fitness, and therefore adaptive plasticity that can benefit these traits in the face of rapidly changing or stressful environmental conditions may play a huge role in the evolution of populations. Future studies should consider investigating variations in mitochondrial biology as another potential mechanism underlying such adaptive developmental plasticity, as it is a link between glucocorticoids, DNA damage, and longevity, and has been recognized as a mediator of life-histories (Speakman et al., 2015).

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

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Data accessibility statement

All data are available in the main text or supplementary materials.

Competing Interests

The authors declare no competing interests.

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Chapter 6: Heat-induced maternal effects shape avian eggshell traits and embryo development and phenotype at high incubation temperatures

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Summary statement: Zebra finch mothers exposed to a mild heat conditioning as juveniles produce embryos whose phenotypes depend upon their incubation temperature as well as their mother's adult environment.

Abstract

Phenotypic plasticity is an important avenue by which organisms may persist in the face of rapid environmental change. Environmental cues experienced by the mother can also influence the phenotype of offspring, a form of plasticity called maternal effects. Maternal effects can adaptively prepare offspring for the environmental conditions they will experience, however, their ability to buffer offspring against environmental stressors as embryos is understudied. Using captive zebra finches, we performed a maternal-offspring environmental match-mismatch experiment utilizing a 2x2x2 factorial design. Mothers were exposed to a mild heat conditioning (38 °C) or control (22 °C) treatment as juveniles, an acute high heat (42 °C) or control (22 °C) treatment as adults, then paired for breeding. The eggs produced by those females were incubated at a hyperthermic (38.5 °C) or optimal temperature (37.2 °C). We found that when mothers were exposed to a mild heat conditioning as juveniles, their embryos exhibited reduced water loss, longer development times, and produced hatchlings with heavier pectoralis muscles when incubated at high incubation temperatures, compared to embryos from control mothers. Mothers exposed to both the mild heat conditioning as juveniles and a high heat stressor as adults produced eggs with a higher density of shell pores, and embryos with lower heart rates during development. However, there was a cost when there was a mismatch between maternal and embryo environment. Embryos from these conditioned and heat stressed mothers had reduced survival at control incubation temperatures, indicating the importance of offspring environment when interpreting potential adaptive effects.

Introduction

Thermal biology drives geographical range limits of a species, and those that cannot rapidly adapt or migrate may experience severely diminished populations or extinction in the face of climate change (Thomas et al., 2006). Populations with slower growth rates, such as those with long generation times, risk extinction before genetic adaptation to the new environment can occur (Chevin et al., 2010). However, phenotypic plasticity (the ability of one genotype to produce multiple phenotypes) may give organisms a faster mechanism of persistence in rapidly changing environmental conditions compared to genetic adaptation. In one form of phenotypic plasticity, the environmental conditions experienced by parents can also affect the phenotype of offspring without alteration of DNA. Such plasticity is known as intergenerational phenotypic plasticity, or maternal effects, when mediated by the mother (Marshall and Uller, 2007). Maternal effects were once thought of as deviations from normal offspring development, but have been recognized for their importance in influencing offspring phenotype and evolutionary processes (Bernardo, 1996). Maternal effects can improve the fitness of offspring in a changing environment by adjusting their phenotype to match the expected environmental conditions (Mousseau and Fox, 1996). These adjustments are often called anticipatory maternal effects (Marshall and Uller, 2007). However, maternal effects may not always be beneficial to offspring fitness. In some cases, maternal effects may increase maternal lifetime fitness while having a detrimental effect on the performance or survival of current offspring (Marshall and Uller, 2007*;* Bernardo, 1996). These are known as selfish maternal effects (Marshall and Uller, 2007*;* Bernardo, 1996). This is in large part because selection acts to maximize fitness of the mother rather than that of the individual offspring, although they are often positively correlated (Bernardo, 1996). Thus, in order for maternal effects to increase offspring fitness, the

environment experienced by the mother must be heterogeneous and an accurate predictor of the environment that will be experienced by offspring.

The strength and direction of maternal effects can be determined in part by the life-history and resources available to the mother. Stressful environments often induce maternal effects at a cost to the mother, imposing a trade-off between self-maintenance and the current reproductive bout (Marshall and Uller, 2007; Sheriff and Love, 2013). Thus, maternal effects that increase offspring fitness are more likely to occur when the plasticity is not costly to the mother, such as when the environmental stressor is mild enough or temporally far enough away from reproduction to not incur maternal trade-offs (Berrigan and Scheiner, 2004). Mothers may also avoid facing trade-offs if they have a tolerance to the stressful conditions via phenotypic plasticity of their own, such as through conditioning hormesis (Calabrese et al., 2007; Costantini and Metcalfe, 2010). In such scenarios, mild stressor exposure adjusts the phenotype of an organism, promoting an increased tolerance to a higher severity of the stressor later on in life, (Calabrese et al., 2007; Costantini and Metcalfe, 2010). Maternal within-generation plasticity may result in offspring with phenotypes better matched to their environments, as mothers with phenotypes that shift due to environmental cues may provide more accurate predictors of future conditions (Kuijper and Hoyle, 2015; Leimar and McNamara, 2015). Therefore, a mother's capacity to promote maternal effects can interact with, and in some cases may be dependent upon, the plasticity of their own phenotype. For example, *Drosophila melanogaster* exposed to variable thermal conditions during early ontogeny had better thermal tolerance than those exposed to high or constant temperatures and also produced offspring with increased thermal performance at constant temperatures, as measured by locomotor performance (Cavieres et al., 2019). However, experiments that incorporate multiple stressor exposure points experienced by

mothers are limited, though such data may be critical in determining how within-generation plasticity influences maternal effects and the stress tolerance of the next generation.

Ambient temperature is an ecologically variable environmental factor that is a strong driver of maternal effects in many species, influencing offspring traits such as dispersal, reproduction, and growth (Burgess and Marshall, 2011; Donelson et al., 2016; Salinas and Much, 2012). Maternal exposure to increased temperatures has also been demonstrated to induce anticipatory maternal effects specifically, shaping offspring phenotypes to optimize their fitness in the ambient temperatures they experience postnatally (Burgess and Marshall, 2011; Crill et al, 1996; Salinas and Much, 2012; Schiffer et al., 2013; Zizzari and Ellers, 2014). For example, threespine stickleback offspring had larger body sizes when reared at the same temperature at which their mothers had previously acclimated to (Shama et al., 2014). However, the bulk of the published research on temperature-induced maternal effects has focused on invertebrates and ectothermic vertebrates, and examine offspring performance postnatally (Shama et al., 2014). As such, the potential for maternal effects to improve embryonic development and survival has been understudied, especially in endotherms.

Embryos of many species have not yet developed the physiological systems necessary to detect environmental cues and adaptively adjust their phenotype (Uller, 2008). Potentially, maternal effects can circumvent this issue by producing offspring pre-conditioned to the otherwise detrimental thermal conditions. Although avian embryos develop outside of the mother in an egg, providing a virtually closed environment, prenatal maternal effects play an important role in many species (von Engelhardt and Groothuis, 2011). Embryos of avian species are generally able to withstand low ambient temperatures for relatively prolonged periods, as parents must leave the nest to forage, leading to temporary cooling of the egg (Conway and Martin,

2000; Mueller et al., 2015; Webb, 1987; DuRant et al., 2013). In contrast, most embryos cannot withstand hyperthermic conditions for very long (Mueller et al., 2015; Webb, 1987). Rising incubation temperatures increases embryonic metabolic demands, resulting in higher oxygen consumption, water loss, and CO_2 build up, eventually leading to desiccation, respiratory or cardiac failure, and death (Mortola, 2009). A high eggshell gas conductance can ameliorate some of these effects by increasing the rate at which oxygen diffuses in and CO_2 diffuses out. Although, this high gas conductance comes with the trade-off of increasing water loss to potentially fatal levels (Mortola, 2009). The rate of gas exchange necessary to meet embryonic metabolic demands is influenced by the pore density and thickness of the eggshell, and therefore these traits can largely impact development, survival, and the environment *in ovo.* Eggshell pore density and thickness differs across species ranges due to adaptation to varying environmental conditions such as temperature, humidity, and altitude, which is thought to occur across many generations (Rahn et al., 1977; Morales et al., 2013). However, if these eggshell characteristics display a degree of plasticity and could be adjusted due to the thermal conditions experienced by the mother, it could in part benefit their offspring when faced with rapid changes in ambient temperature in a single generation.

In this study, we used a captive colony of zebra finches (*Taeniopygia castanotis*) to investigate whether maternal exposure to heat induces maternal effects in their offspring's phenotype and whether those changes are mediated through eggshell characteristics. Using a 2 x 2 x 2 factorial design, we examined how the maternal thermal environment influences embryo physiology, development, and survival at optimal and high incubation temperatures. To test this, we exposed juvenile females to a mild heat conditioning (38 °C) or control (22 °C) temperature treatment every other day for 28 days (Fig. 1). This conditioning protocol can be considered a

mild heat stressor because the temperature of 38 °C is just above the upper critical temperature of the thermoneutral zone for the species (between 34.9 and 37.5 °C) as determined by (Wojciechowski et al., 2021), but has also been shown to not increase blood oxidative damage (Costantini et al., 2012). In the wild, even some of the hottest areas in Australia (such as near Alice Springs and Telfer) historically experience less than 28 days a month with temperatures above 35 °C in the summer (Australian Government Bureau of Meteorology). However, the number of days per month with such high temperatures is increasing and is predicted to increase even further as a result of climate change (Conradie et al., 2020). As adults, the finches were then exposed to either a high heat stressor (42 °C) or control temperature (22 °C) treatment for 3 consecutive days (Fig. 1). These females were paired to breed with non-experimental males, and the resulting eggs laid were incubated for the duration of embryonic development at either a control temperature (37.2 °C), or a high temperature (38.5 °C), the latter of which has been shown to decrease hatching success in this species (Wada et al., 2015). We then measured several developmental and physiological parameters of offspring such as embryonic heart rate and development time, change in egg mass during incubation, and hatchling morphology. We also measured eggshell pore density and thickness to determine whether these characteristics can be influenced by the thermal conditions experienced by the mother, and whether this plasticity is associated with changes in offspring phenotype. We hypothesized that exposure to a mild heat conditioning treatment as juveniles would induce an anticipatory maternal effect, resulting in mothers producing offspring that are better suited to cope with high incubation temperatures, regardless of the thermal conditions mothers were exposed to as adults prior to breeding. Specifically, we predicted that these embryos would exhibit less water loss, lower heart rates, higher organ masses, and eggshells with increased pore density when compared to embryos from

juvenile control mothers. We also predicted that non-conditioned mothers exposed to an acute and high intensity thermal stressor in adulthood prior to breeding would induce a selfish maternal effect, producing offspring that have reduced body and organ masses and survival regardless of incubation temperature, and increased heart rates and water loss at high incubation temperatures.

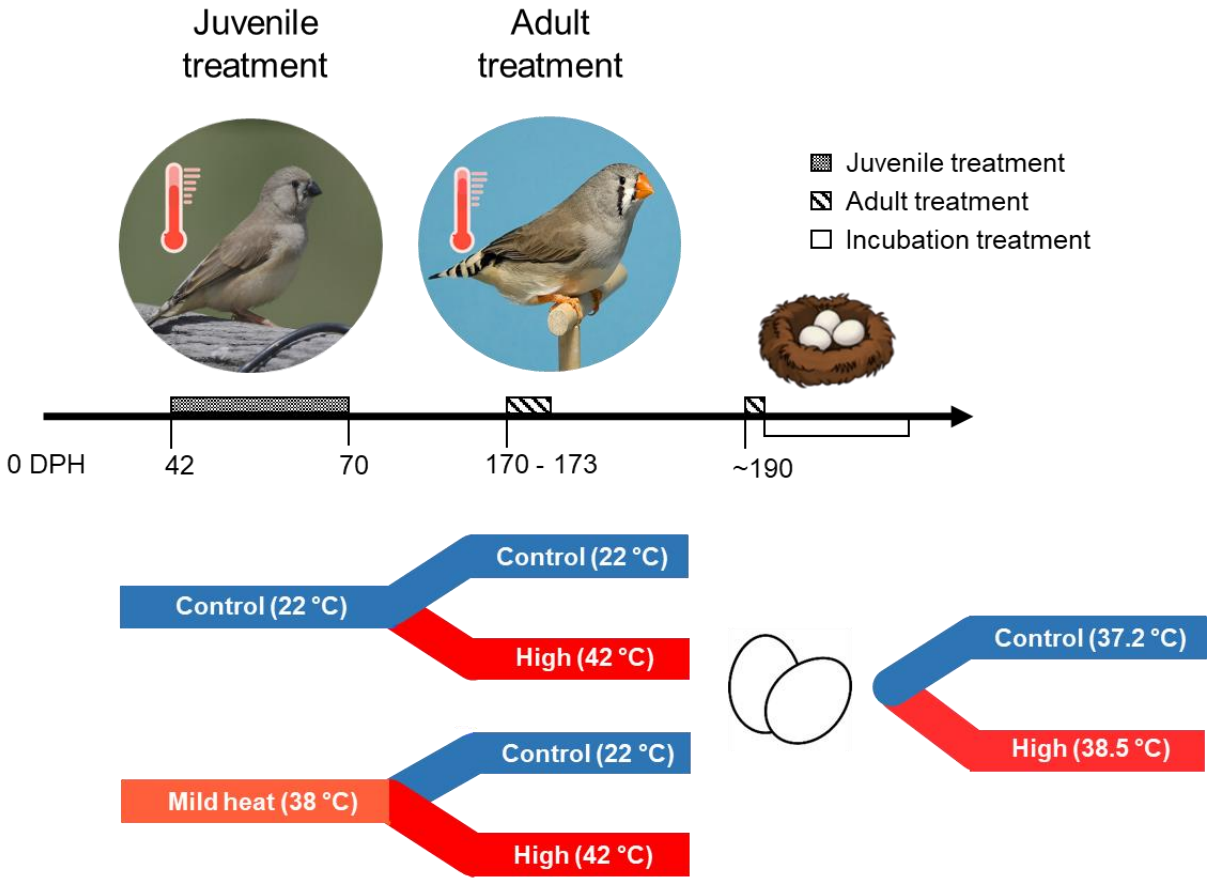

Fig. 1. Schematic representation of the experimental timeline and depiction of treatment groups. Beginning at 42-45 days post-hatch (DPH), females were exposed to either a mild heat (38 °C) or control (22 °C) treatment over a 28-day period. In adulthood, starting at 170-173 DPH, these females were then exposed to either a high heat stressor (42 °C) or control temperature (22 °C) over a 3-day period in a 2x2 factorial design. Several weeks later, the eggs produced by these females were removed from the nest and artificially incubated at an optimal (37.2 °C) or high (38.5 °C) incubation temperature.

Materials and Methods

Animal diet and husbandry

Experimental females used in this experiment were obtained from our captive colony of zebra finches (*Taeniopygia castanotis*) at Auburn University. The details of the husbandry and experimental treatment of the mothers used in this study have been previously published (Hoffman et al., 2018). Briefly, female nestlings from 26 breeding pairs were used in this study. The finches received *ad libitum* mixed seeds (Kaytee Supreme (finch), Chilton, WI), water, grit, and cuttlefish bone as well as a once weekly supplement of vitamin drops and spinach and daily supplement of egg mixture (hard-boiled chicken egg, white bread, and cornmeal). Upon reaching 10 days post-hatch (DPH), nestlings were temporarily removed from the nest to be weighed and metal ID bands with a unique four-digit number were attached to their leg. Juveniles were separated from their parents at 39-43 DPH and kept with other juveniles until the start of treatment.

Juvenile treatment

A total of 48 females underwent the juvenile conditioning treatment, following a protocol that has been previously established (Costantini et al., 2012) and used in our laboratory (Hoffman et al., 2018). The oldest offspring in each nest was randomly assigned to either the control or mild heat treatment group, after which we alternated treatment group assignments while accounting for sex in order to balance treatments within each nest. At 42-45 DPH, half of the juvenile females began the mild heat treatment and were exposed to a temperature of 38 °C on every other day, 3 hours per day, for 28 days in a brooder (TLC-50; Brinsea Products Inc., Titusville, FL) as previously described (Costantini et al., 2012*;* Hoffman et al., 2018). For this species, body temperature begins to increase at ambient temperatures higher than approximately 35.9 °C, leading to mild hyperthermia (Wojciechowski et al., 2021). This increase in body temperature limits water loss by creating a thermal gradient allowing for better heat dissipation

and may initiate stress responses such as glucocorticoid secretion and heat shock protein synthesis (Feder and Hofmann, 1999; Wingfield et al., 1998). The other half of the juvenile females were used as a control group, and were placed into brooders in the same manner as the mild heat group, but instead set to our general aviary room temperature of 21-23 °C. Although this temperature is below the species thermoneutral zone, it was used as a control treatment because it is the temperature at which they, as well as previous generations, were reared. When undergoing treatment bouts, we placed 3–7 finches in brooders beginning between 8:30 and 9:30 a.m. local time. While in the brooders, finches received *ad libitum* cold water that was replaced at the first- and second-hour mark of the 3-hr treatment period. When not in the brooder undergoing treatment, all birds were housed in general aviary temperature conditions (21-23 °C) and kept in single sex tower cages (1-3 birds per cage) and received *ad libitum* of the same diet described above. Following the end of the 28-day juvenile treatment period, birds were housed in these same conditions until the start of the adult stage treatment at 170-173 DPH.

Adult treatment

At 170-173 DPH, half of the females from each juvenile stage treatment were exposed to a high heat stress treatment of 42 °C for 3 hours a day, for 3 consecutive days as previously described (Costantini et al., 2012*;* Hoffman et al., 2018). This temperature is well above the upper critical temperature of the thermoneutral zone and has been shown to cause blood plasma oxidative damage in this species (Wojciechowski et al., 2021; Costantini et al., 2012). The remaining females from each of the juvenile stage treatment groups served as the adult-stage control group and were exposed to room temperature (21-23 °C) in the same manner as above. This 2 x 2 factorial design resulted in four maternal treatment groups that were the different

combinations of juvenile and adult treatment (Control-Control [CC]: n = 13, Control-High [CH]: n = 14, Mild-Control [MC]: n = 11, and Mild-High [MH]: n = 9).

Female pairing

Females were paired with non-experimental males and on the day of pairing, females first underwent a single additional 3-hour bout of their respective adult-stage treatment (control or high heat) as described above. Pairs were housed in the same breeding conditions and received the same diet as previously described. A total of 43 females (*n*: CC – 12, CH – 12, MC – 10, MH – 9) successfully produced eggs that underwent the incubation stage treatment.

Egg collection and incubation treatment

After pairing, nests were checked for eggs daily at approximately 10:00 a.m. local time. Once laid, eggs were removed from the nest and marked with a unique ID number using a fine tip permanent marker. The first egg laid in the clutch was replaced with a false egg made of modeling clay (Crayola Model Magic [white], Easton, PA) after removal, while any subsequent eggs removed were not replaced. The first egg laid was randomly assigned to an incubation treatment (a control or high incubation temperature) and following that the first laid egg of each subsequent nest was assigned a treatment group in an alternating manner to balance treatment allocation among the first eggs of the clutch. After the first egg, treatment group assignments alternated within each clutch to balance laying order and nest of origin between incubation temperature treatments. Therefore, in general, there were multiple embryos in both treatment groups for each female.

Newly laid eggs were transported to the lab where they were weighed and then placed into one of two incubator treatments, representing a control or high incubation temperature.

Those assigned to the control group (*n* = 125 embryos) were transferred into an incubator (Brinsea Octagon 20 Advance EX incubator, Titusville, FL) set to 37.2 °C for the entirety of embryonic development. This temperature was chosen as it is very close to the average incubation temperature of captive zebra finches (Zann and Rossetto, 1991) and was found to be optimal for the species when considering both hatching success and post-hatch survival (Wada et al., 2015). Individuals assigned to the high incubation temperature (*n* = 106 embryos) were placed into an incubator set to a temperature of 38.5 °C, which is slightly above the range of average incubation temperatures for wild and laboratory kept zebra finches (Zann and Rossetto, 1991), for the entire incubation period. In this species, an incubation temperature of 38.4 °C can result in lower hatching success overall and in lower overall and lean body mass in male offspring during their postnatal growth period (Wada et al., 2015; Wada et al., 2018) and therefore considered outside the thermal optimum. Both incubators were set to maintain a relative humidity of 55%. Incubators were calibrated to the desired temperature using a reference thermometer placed at level with the eggs. Approximately three times per week, incubators were taken off of their rockers and allowed to sit level for at least one hour before reading the thermometer to determine temperature accuracy. The average measured temperature in control and high temperature incubators were 37.13 °C ± 0.51 and 38.45 °C ± 0.42 (mean ± standard deviation), respectively. No incubator replicates were used, however both incubators were distanced only ~20 centimeters apart and kept in a temperature-controlled room with no foot traffic.

Heart rate measurements

Embryonic heart rates were measured using the Buddy® digital heart rate monitor (Avian Biotech, Vetronic Services, Abbotskerswell, Devon, UK). The Buddy® digital heart rate monitor

uses a non-invasive method which quantifies heart rate by the amount of infrared light absorbed by the pulsing embryonic blood. The Buddy® monitor has been used in a variety of studies on oviparous embryos, often examining the effects of incubation temperature on reptilian embryos (Du et al., 2009; Hulbert et al., 2017; Hall and Warner, 2018). More specifically, the Buddy® monitor has also been used in studies where poultry and turkey embryos were exposed to non-optimal temperatures, as well as studies on other avian species using a variety of stimuli (see Sheldon et al., 2018). More recently, a thorough investigation using the Buddy® system has been done on wild zebra finch embryos (Sheldon et al., 2018).

Heart rate measurements were recorded repeatedly at two different time points during an individual's embryonic development, first at embryonic day (ED) 4, which is at ~28.9% of development for embryos in the control temperature incubator and ~29.4% of development in the high temperature incubator. The second measure was taken at ED 11 for embryos in the control incubator and at ED 10 for embryos in the high incubator, which is ~79.6% and ~73.5% of development respectively. Heart rate data were collected between 2:00 and 5:00 p.m. local time to account for any circadian variation.

When measuring heart rates, eggs were individually removed and placed horizontally (pointed tip facing digital display) on the measuring platform of the monitor before closing the lid. A timer was started as soon as the egg was removed from the incubator, and the amount of time (s) that the embryo had been out of the incubator at each heart rate (bpm) measurement was recorded. This value was used in the analysis to account for the decrease in heart rate when eggs were removed from the incubator and began to cool. Heart rates were recorded every 10 seconds after the first recording until the egg had been outside of the incubator for ~70 seconds. If reliable heart rates could not be picked up by the monitor by the time the egg had been out of the

incubator for ~40 seconds, the egg was flipped 180° on the platform. Measurements were considered reliable when the output values were consistent and continuous, with the “heart icon” on the digital display blinking. The eggs did not remain inside the monitor for longer than ~90 seconds in total. The heart rate measurement taken between the 50 and 60 second mark was used in statistical analyses. For day 4 measurements, eggs were immediately placed back into their respective incubator after heart rates had been recorded. For day 10/11 measurements, each egg was weighed after recording the heart rate to calculate mass loss before being returned to incubators. Eggs did not remain outside of the incubators for longer than ~240 seconds total while measuring heart rate and egg mass. The order in which eggs were removed from the incubator for measurements was also recorded to be taken into account in the statistical analysis. When no heart rate signal could be found using the monitor, eggs were candled using a flashlight to determine infertility or mortality. If an egg was found to have a crack due to handling or collisions inside the incubator, the heart rate was not measured and that individual was not used in survival analysis. When an embryo was found to be dead and had no cracks in the shell, the developmental stage (very early, early, mid, late, very late, pipping) at which mortality occurred was estimated and recorded for later use.

Organ measurements

All incubators were checked a minimum of three times daily for potential hatchlings. When a hatchling was found, it was removed from the incubator, weighed, and then euthanized via isoflurane inhalation as per approved protocol. Dissected tissues included the residual yolk sac, heart, and pectoralis muscle, and the tissues were weighed prior to freezing. After removal, all dissected tissues were then flash frozen in liquid nitrogen before being placed in a -80° C freezer, while the carcass was placed in a freezer at -20° C for later use.

Eggshell measurements

Pore density was determined using a protocol refined for songbird eggs by Stein and Badyaev (2011) with some modification. Briefly, three pieces of shell from each egg were chosen representing the blunt end, equator, and pointed end of the egg. These pieces were then placed in a biopsy case and incubated in hot NaOH bath for 3 minutes to remove the eggshell membrane. Following this, the shells were rinsed in ddH2O, then dried in an incubator set to 41 °C. Once dry, the thickness of the shell was measured using a micrometer (Mitutoyo, cat number 395-371-30, 0.001mm resolution) to the nearest 0.001mm. Shells were then incubated in 2.5% nitric acid for 12 seconds, followed by a rinse in ddH_2O. The shells were dried once again in an incubator set to 41 °C. Once dry, the number of eggshell pores were counted using a Nikon Eclipse microscope. One researcher measured the thickness, and another researcher counted all the pores for all samples. Both researchers were blind to the treatment groups from which the eggshells came.

Statistical analyses

All data used for analyses are available in the supplemental files (Data S1 and Data S2) and stored in the *Dryad* digital data repository. Statistical analysis was done using RStudio (RStudio Team, 2020) utilizing the packages “nlme” and “survival” and graphs were constructed using the “ggplot2” and “survminer” packages. Statistical tables were constructed using the “tab_model” function in the package “sjPlot”. Statistical significance was determined as $P \leq 0.05$, and all models included the maternal ID (“FID” or “nestid”) as a random effect to account for multiple eggs originating from the same mother. To examine the effects of our experimental treatments on dependent variables we first ran initial “full” models that included the juvenile*adult*incubator treatment interaction term and the lower order two-way interaction

terms, as well as predictors that were believed likely to have a large effect size or plausible biological effect on some aspects of the model. Predictors remained in the model regardless of significance, while non-significant interaction terms were removed when lower order conditional main effects, lower order interaction terms, and other predictors were also non-significant. Assumptions of linearity of the data were tested by plotting the residuals versus fitted values. Assumptions of normality of residuals were tested via histogram plots of the data for linear models. For linear models, regression coefficients were used for estimates of mean change in response variables, while odds ratios were used for estimates in generalized linear regression models. Estimates of uncertainty were represented via 95% confidence intervals, calculated using the functions "intervals" and "confint" for linear regressions and generalized linear mixed-effects models respectively.

Egg mass and heart rate

Linear mixed-effects models (*LMM*) were used to analyze effects on all egg mass, heart rate, and hatchling mass variables. To test the effect of maternal treatment on initial egg mass (mass on the day egg was laid and prior to incubation treatment), we used a model with juvenile treatment, and adult treatment as fixed factors, lay order (the sequential order in which the egg was laid for each female) as a covariate, as well as a maternal juvenile*adult treatment interaction term. Incubation treatment was not included in this egg mass analysis, as the initial egg mass was taken before being placed in the incubator. The change in egg mass over the first ~77% of embryonic incubation was analyzed using a model with juvenile, adult, and incubation treatment as fixed factors, a juvenile*incubation treatment interaction term as well as the embryonic heart rates at both ~30% and ~77% of embryonic development included as covariates. The effect of maternal and incubation treatments on embryonic heart rates at ~30% development

was analyzed using juvenile, adult, and incubation treatment as fixed factors, as well as the two-way juvenile*adult interaction term. The effect of maternal and incubation treatments on embryonic heart rates at ~77% development was analyzed using juvenile, adult, and incubation treatment as fixed factors, as well as the two- and three-way interaction terms, and initial egg mass as a covariate. A further post-hoc analysis was done using an *LMM* with a categorical variable "fulltreatment" representing the eight combinations of juvenile, adult, and incubation treatments as a fixed factor, and initial egg mass as a covariate. The coded variable represents the eight distinct treatment groups resulting from the 2x2x2 factorial design and allows for post-hoc pairwise comparisons.

Embryonic development time and hatchling organ mass

The effect of maternal juvenile and incubation treatments on embryonic development time was analyzed using an *LMM* with juvenile, adult and incubation treatment as fixed factors, a juvenile*incubation treatment interaction term, as well as the embryonic heart rate at ~77% of embryonic development included as a covariate. A further post-hoc analysis was done using a *LMM* which included a categorical treatment variable representing the four combinations of maternal juvenile and incubation treatment as a fixed factor, adult treatment as a fixed factor, as well as the embryonic heart rate at ~77% of embryonic development included as a covariate. Pectoralis mass was analyzed using a *LMM* with juvenile, adult, and incubation treatment as fixed factors, a juvenile*incubation treatment interaction term, hatchling body mass, and incubation time as covariates. A further post-hoc analysis was done using a *LMM* which included the juvenile*incubation categorical treatment variable (see above) as a fixed factor, adult treatment as a fixed factor, and hatchling body mass and incubation time as covariates. The effect of maternal and incubation treatments on body (total hatchling mass prior to euthanasia)

mass of hatchlings was analyzed using a *LMM* which included juvenile, adult, and incubation treatment as fixed effects, their two- and three-way interaction terms, initial egg mass, incubation time, and embryonic heart rate at ~77% of embryonic development as covariates. The model examining the effects of treatments on hatchling residual yolk (the mass of yolk internalized from egg stores that were present at hatch) and heart mass included juvenile, adult, and incubation treatment, their two- and three-way interaction terms, as well as hatchling total body mass as a covariate.

Eggshell characteristics

We used *LMMs* for all models to determine the effect of maternal and incubation treatments on eggshell characteristics. The difference in eggshell pore density between embryos that lived and died was analyzed using a model which included survival (whether the embryo died during development or successfully hatched) as a factor, initial egg mass as a covariate, and their two-way interaction term. The difference in eggshell thickness between embryos that lived and died was analyzed using a model which included survival as a factor, initial egg mass as a covariate, and their two-way interaction term. We analyzed the effect of maternal treatment on eggshell pore density using a model which included juvenile and adult treatment as fixed factors, as well as the two-way interaction term, and egg mass as a covariate. A further post-hoc analysis was done using a *LMM* which included a juvenile*adult categorical treatment variable (see above) as a fixed factor, and egg mass as a covariate. We analyzed the effect of maternal treatment on eggshell thickness using a model which included juvenile and adult treatment as fixed factors, as well as their two-way interaction term, and egg mass as a covariate. A further post-hoc analysis was done using a *LMM* which included a juvenile*adult categorical treatment variable as a fixed factor, and egg mass as a covariate.

Embryo survival

The effect of incubation treatment on embryonic survival was analyzed using a generalized linear mixed-effects model (*GLMM*) which included juvenile, adult, and incubation treatment as fixed effects, as well as the two- and three-way interaction terms. The data was then subset by incubation treatment to examine the effects of maternal treatments within different incubation temperatures using *GLMs* which included the maternal juvenile*adult categorical treatment variable as a fixed factor. Kaplan-Meier curves were generated using models which included the maternal juvenile*adult categorical treatment variable as a fixed factor.

Results and Discussion

For avian embryos, the demand for oxygen generally increases as temperatures rise, leading to an increased production of CO_2 as well as higher water loss through diffusion across the eggshell (Mortola, 2009). While the ratio of CO_2 lost to O_2 gained is between 0.70 – 0.75 in the avian egg, the O_2 molecule also has 27% lower mass, meaning that the mass exchanged due to the diffusion of these molecules is nearly equivalent (Mortola, 2009). Therefore, during the course of incubation, the change in mass of the embryo can be attributed almost entirely to water loss. In our experiment we used the change in mass as an indirect measure of water loss. When we compared the water loss among eggs produced by control mothers, we saw an expected pattern. Embryos from juvenile control mothers lost 5.63×10^{-2} g (±0.047, ±95% CI) more water at a higher incubation temperature compared to embryos incubated at the control incubation temperature (Fig. 2) (Linear mixed model [LMM]: $t = -2.50$, d.f. = 22, $P = 0.0204$, random effect (SD) = 1.14×10^{-6}). However, mothers who were exposed to the mild heat conditioning as juveniles produced embryos that were seemingly more resistant to water loss at high ambient temperatures. Embryos from these heat-conditioned mothers lost similar amounts of water at

higher incubation temperatures compared to embryos incubated at a control temperature (Fig. 2) (LMM: t = 0.223, d.f. = 22, P = 0.826, random effect (SD) = 1.42 x 10^{-6}). In contrast, the thermal treatment mothers received as adults approximately three weeks before breeding had no effect on the water loss during incubation. This effect of maternal treatment on water loss cannot be attributed to differential egg size alone, as there were no differences in initial egg mass among the groups (Table S1).

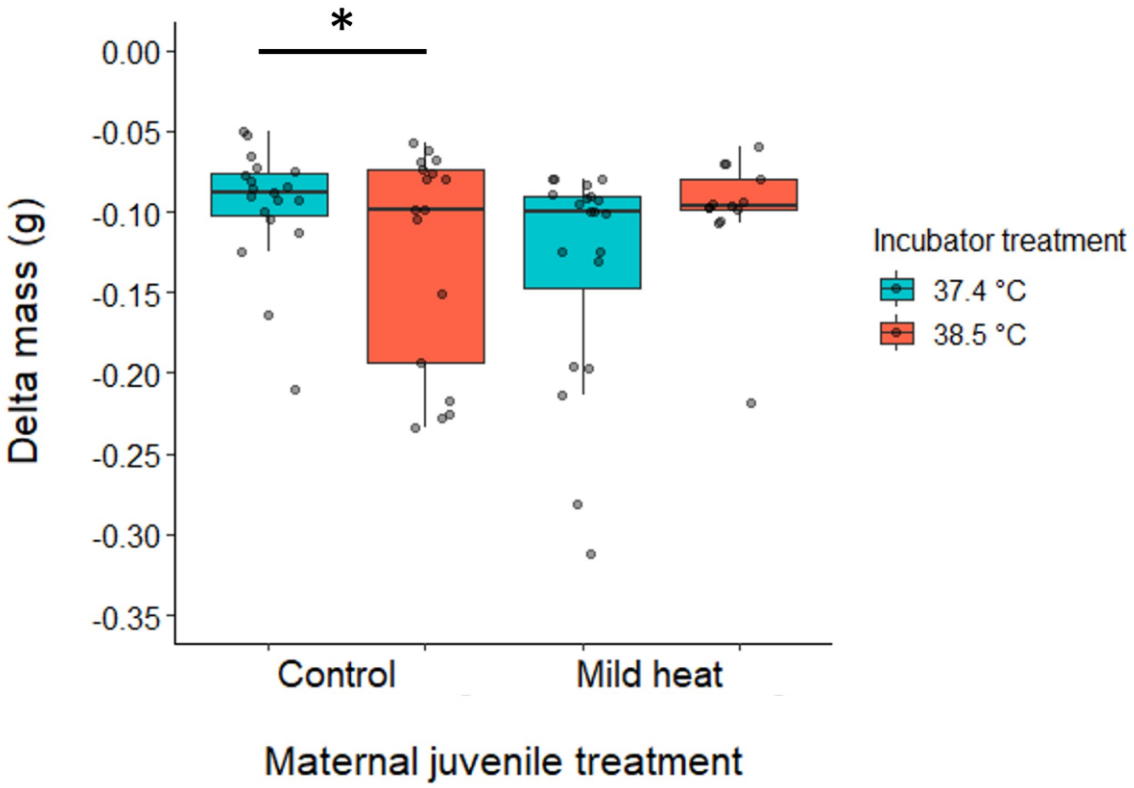

Fig. 2. At high temperatures, embryos showed no increase in egg mass loss when produced by mothers exposed to a mild heat conditioning as juveniles. The change in egg mass (g) occurring from the day the egg was laid until approximately 77% of the incubation period. The x-axis denotes the treatment mothers received as juveniles, either a control (22 °C) or mild heat (38 °C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2 °C) or high incubation temperature (38.5 °C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers). Significance indicated as follows for all graphs *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

At standardized humidity, differences in embryonic water loss can be attributed in part to metabolic rate. Therefore, we investigated how maternal heat exposure shapes the metabolic rate of offspring as embryos. To do this, we measured embryonic heart rate – which is frequently used as a proxy for metabolic rate in birds and reptiles due to its strong correlation with oxygen consumption – at ~30% and ~77% of embryonic development (Du et al., 2009; Sheldon et al., 2018). We found that exposure to high incubation temperatures increased heart rates of embryos at ~30% of development across all samples, but there was no difference seen due to the mother's treatment at this temperature (Table S1). However, at the control incubation temperature, embryos from mothers that experienced the mild heat as juveniles (Mild-Control) had heart rates 13.1 bpm (±11.4, ±95% CI) lower than those produced by mothers with no prior heat exposure (Control-Control) (Fig.3a) (LMM: t = -2.28, d.f. = 223, P = 0.0237, random effect (SD) = 1.58). Later on in embryonic development (at ~77%), we again observed a maternal effect on heart rate. When incubated at the high temperature, embryos from mothers exposed to both heat treatments (Mild-High) had significantly lower heart rates than embryos from all other maternal treatment groups (Fig. 4b) (Table S2 - LMM: d.f. = 142 all $P < 0.01$, random effect (SD) = 4.98). Reduced heart rates could act as an adaptive maternal effect, especially at high incubation temperatures. As heart rates increase with ambient temperature, so does the risk of cardiac arrhythmia and arrest (Mueller et al., 2022). Zebra finch embryos already exhibit exponential increases in heart rate and oxygen consumption late in development, which can make them potentially more susceptible to death as a result of hyperthermic conditions at this time (Vleck et al., 1979; Pearson et al., 1999). Therefore, lower heart rates as exhibited by embryos from the Mild-High mothers may increase the chance for survival at high temperatures. As expected, there was a negative correlation between the embryonic heart rate at ~77% of embryonic development and

change in egg mass during development across all samples. For each 1 beat per minute (bpm) increase in heart rate, we saw a 4.76 x10^{-4} g (± 3.31x10^{-4}, ±95% CI) increase in mass (water) loss (LMM: t = -2.92, d.f. = 35, P = 0.006, random effect (SD) = 1.15 x 10^{-6}). However, this pattern did not hold true for all treatment groups. At control incubation temperatures, embryos from mothers exposed to both heat treatments (Mild-High) exhibited the aforementioned increase in water loss as heart rates increase (LMM: t = -2.56, d.f. = 11, P = 0.0264, random effect (SD) = 1.85 x 10^{-6}). However, at high incubation temperatures, embryos from this same group showed no significant increases in water loss as heart rates increased (LMM: t = -1.04, d.f. = 6, P = 0.339, random effect (SD) = 3.22 x 10^{-6}). While these decreases in water loss and heart rate at high incubation temperatures exhibited by embryos from heat-conditioned mothers may represent an adaptive change in physiology, reducing the risk of death via desiccation or cardiac failure, such physiological changes may have trade-offs with other components of embryonic development.

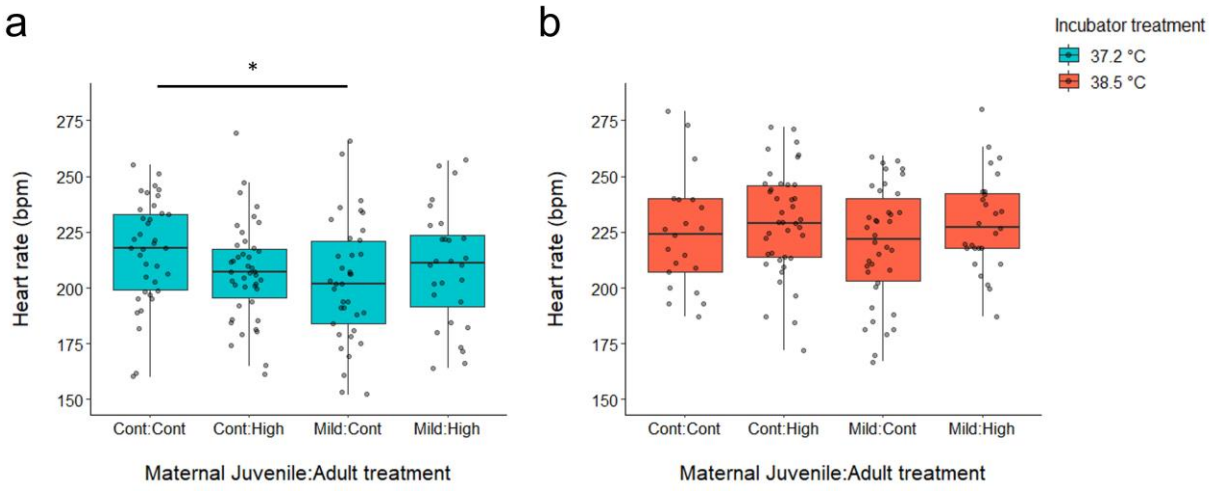

Fig. 3. Embryos produced by mothers exposed to the mild heat conditioning as juveniles had lower heart rates, but only at the control incubation temperature. (a). The heart rates of embryos in the control incubation treatment at approximately 30% of the development period. (**b).** The heart rates of embryos in the high incubation treatment at approximately 30% of the development period. For each group on the x-axis, the first word denotes the treatment the mothers received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2 °C) or high incubation temperature (38.5 °C).

Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers).

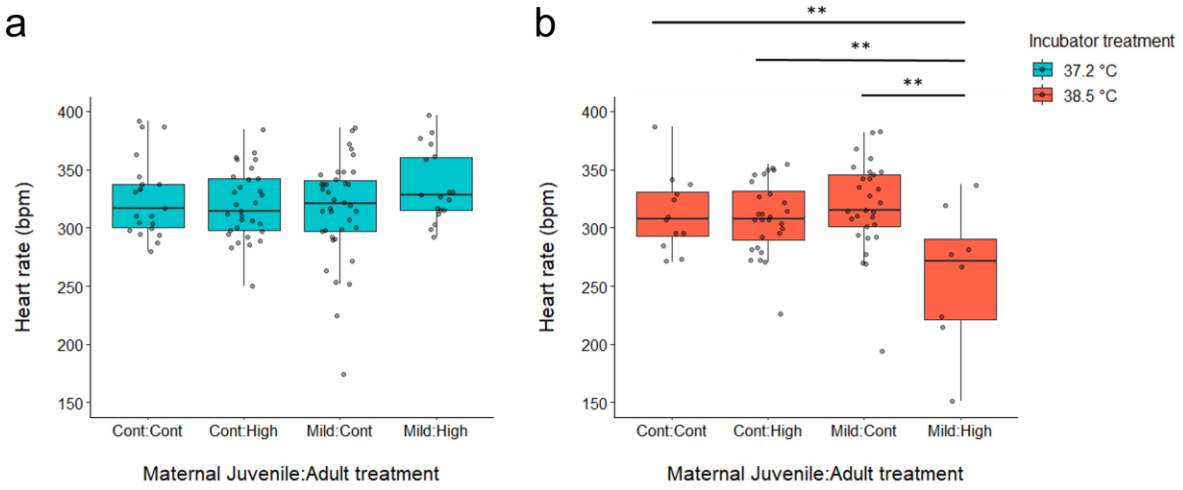

Fig. 4. Embryos produced by mothers exposed to the mild heat conditioning as juveniles and high heat stressor as adults had lower heart rates, but only at the high incubation temperature. (a). The heart rates of embryos in the control incubation treatment at approximately 77% of the development period. (**b).** The heart rates of embryos in the high incubation treatment at approximately 77% of the development period. For each group on the x-axis, the first word denotes the treatment the mothers received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat). The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2 °C) or high incubation temperature (38.5 °C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers).

Non-optimal incubation temperature can result in changes of embryonic development time and/or differential hatchling morphology (Wada et al., 2015; Wada et al., 2018, Gurley et al., 2018; Rubin et al., 2021). For many avian species including the zebra finch, increases in egg incubation temperature result in decreased incubation periods (Wada et al., 2015). In such situations, embryos may prioritize development of essential organs such as the brain and heart at the expense of others (Mortola, 2009). Here, maternal juvenile treatment increased embryonic development time, however this effect was dependent on the incubation temperature the embryos were exposed to (Table S3). As expected for embryos produced by juvenile control mothers, those that were incubated at the high temperature had a development time 0.435 days (±0.451,

±95% CI) shorter than those incubated at the control temperature (Fig. 5a) (LMM: t = -1.94, d.f. = 47, P = 0.058, random effect (SD) = 0.694). In contrast, embryos produced by mothers exposed to the mild heat conditioning as juveniles had development times 0.974 days (±0.815, ±95% CI) longer than embryos from juvenile control mothers when incubated at high temperatures. (Fig. 5a) (LMM: MH-CH: t = 2.40, d.f. = 47, P = 0.0202, random effect (SD) = 0.719). These relatively longer development times demonstrated by embryos from conditioned mothers, even at high incubation temperature, were similar to development times of embryos incubated at the optimal temperature (Fig. 5a) (CC-MH: β = 0.512, t = 1.42, d.f. = 47, P = 0.162, random effect (SD) = 0.694). As expected, there was a significant negative correlation between development time and embryonic heart rate at ~77% of embryonic development, a pattern similar to that demonstrated in zebra finches and other species (Sheldon and Griffith, 2018; Amos and Tazawa, 1999; Hiroshi, 2005). More specifically, we also saw that the longer development times at high incubation temperatures exhibited by embryos produced by heat conditioned mothers was correlated with their reduced heart rates at ~77% of embryonic development (LMM: t = -2.35, d.f. = 25, P = 0.027).

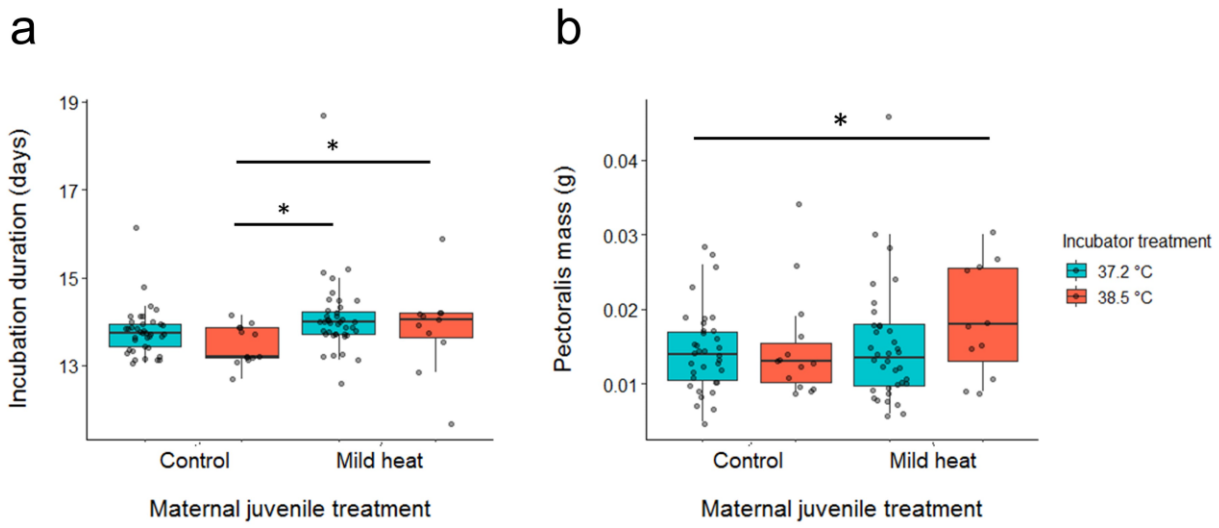

Fig. 5. Embryos produced by mothers exposed to mild heat as juveniles had longer development durations and heavier pectoralis muscles at hatch. (a). The total incubation duration (days) of embryos developed at different temperatures. The x-axis denotes the treatment mothers received as juveniles, either a control (22 °C) or mild heat (38 °C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2 °C) or high incubation temperature (38.5 °C). (**b).** The total weight (g) of offspring pectoralis muscles after hatching. The x-axis denotes the treatment mothers received as juveniles, either a control (22 °C) or mild heat (38 °C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2 °C) or high incubation temperature (38.5 °C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers).

In some scenarios, slow growth of avian embryos is associated with increased individual quality that affects lifetime fitness (Ricklefs et al., 2017). However, there are potential trade-offs as longer development times also increase mortality risks such as by adverse weather events or predation (Ricklefs et al., 2017). While slower embryonic development utilizes more overall energy, the rate of energy consumption is lower (Ricklefs et al., 2017; Rahn and Ar, 1974). This reduced energy consumption rate can explain why the longer development times exhibited by embryos from heat conditioned mothers may be an adaptive maternal effect at high incubation temperatures. Late in avian embryonic development, metabolic demands are at their highest and the risk of death due to hypoxia, water loss, and CO_2 build up is increased (Mortola, 2009). Therefore, avoiding further increases in metabolic demands with increases in temperature during

this time could lower mortality risks. Longer development times may also have beneficial implications for fitness by reducing damage at a cellular level. For example, in some avian species there is a negative correlation between embryonic development time and damage (lipid peroxidation and DNA breakage) suggesting a trade-off between embryonic development rate and oxidative damage, most likely mediated by metabolic rate (Tsunekage, 2015; Tsunekage and Ricklefs, 2015). It has been suggested that oxidative stress is a key mediator of life-history trade-offs by influencing traits such as reproduction and survival, and so avoiding such oxidative insults as embryos by increasing development time may increase lifetime fitness (Costantini, 2008; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010).

Interestingly, the differential developmental patterns observed between treatment groups were also associated with morphology differences at hatching. While there was no effect of maternal treatment on hatchling body, heart, or residual yolk mass, we observed that hatchlings produced by mothers who were exposed to the mild heat conditioning as juveniles had heavier pectoralis muscles when incubated at a high temperature (Table S3). These hatchlings had pectoralis muscles 5.03 $\times 10^{-3}$ g ($\pm$4.91 $\times 10^{-3}$, $\pm$95% CI) heavier than hatchlings produced by juvenile control mothers incubated at a control temperature (Fig. 5b) (LMM: pectoralis: t = 2.05, d.f. = 61, P = 0.0450, random effect (SD) = 5.94 x 10^{-7}). The pectoralis muscles can act as an invaluable thermoregulatory organ in birds, as they are the most essential site for shivering thermogenesis which allows birds to maintain body temperature at ambient temperatures below their thermoneutral zone (Dawson and O'Connor, 1996; Hohtola, 2004; Marsh and Dawson, 1989; Cooper, 2002). Thus, while increased pectoralis muscle mass is associated with adaptations to low ambient temperatures, it's interesting that in this study we saw higher masses in response to increased temperatures experienced by mothers and embryos. However, the

heavier pectoralis muscles in hatchlings may still represent an adaptive change. In chickens, increased incubation temperatures have been demonstrated to result in higher hatchling pectoralis mass, as well as a greater thermotolerance later in life (Collin et al., 2007; Piestun et al., 2013a; Piestun et al., 2013b). Larger pectoralis muscles may also give individual birds an advantage in poor conditions, outside of thermoregulatory effects. An increase in pectoralis muscle without an increase in total body mass (as seen in this study) can improve flight performance and efficiency in birds (Verspoor et al., 2007), which could be adaptive in poor conditions, potentially by increasing predator avoidance capabilities or allowing increased foraging activity levels when food availability is low or unpredictable (Lima, 1993; Witter et al., 1994; Bautista et al., 1998; Noakes et al., 2020). Furthermore, if offspring are forewarned of poor post-hatch conditions via the environment experienced by their mothers and their own pre-natal environment, increased pectoralis muscles may be adaptive by providing proteins reserves to persevere during times of food shortage (Bize et al., 2007). The increased pectoralis mass suggests a potential adaptive maternal effect, as the higher mass and longer development times at high incubation temperatures was not at the cost of reduced overall body, heart, or residual yolk mass (Table S4). However, we cannot rule out that this change in embryo phenotype could incur a cost that only becomes apparent later in life, such as reduced longevity or reproductive output in adulthood. Whether such trade-offs occur may also depend on the thermal environment the offspring would experience post-hatch. Therefore, future experiments examining potential long-term costs or benefits of such maternal effects would do well to utilize multiple post-hatch temperature regimes when evaluating offspring performance.

The underlying mechanisms through which maternal thermal experience influenced embryonic heart rates, development times, and pectoralis mass as seen in this experiment is

unknown, however one potential candidate is through maternal hormone deposition in the egg. Hormone-mediated prenatal maternal effects have been well documented in avian species for their ability to influence development and signal environmental conditions to offspring (Groothuis et al., 2005). Moreso, the amount of steroid hormones a mother deposits in the egg yolk can be influenced by the environmental conditions she experiences (Groothuis et al., 2005). For example, laying hens exposed to a moderate heat challenge (30 °C) for 5 weeks produced eggs with higher concentrations of testosterone, progesterone, and estradiol which influenced hatchling quality and growth (Bertin et al., 2013). Furthermore, increased exposure to steroid hormones *in ovo* has been demonstrated to alter the same embryo phenotypic traits measured in this study. For example, Rock pigeon (*Columba livia*) embryos with experimentally elevated yolk androgen levels exhibited higher heart rates during development and stimulated prenatal growth (Wang et al., 2023). With regards to incubation length, a study showed that zebra finch eggs injected with testosterone had longer embryonic development times than control eggs (Von Engelhardt et al., 2006). Increased embryonic steroid hormone exposure can also influence pectoralis mass, as European starlings (*Sturnus vulgaris*) exposed to experimentally elevated yolk corticosterone as embryos had heavier pectoralis muscles and improved flight performance as fledglings (Chin et al., 2009). Therefore, the alterations in embryonic heart rates, development times, and pectoralis mass from mothers exposed to the thermal treatments, even when incubated at high temperatures, may be the result of differences in circulating steroid hormones in mothers as a result of their prior thermal exposure.

Differential development patterns may not only be attributed to metabolic rate, but also to eggshell traits. Lower pore density and thicker eggshells decrease gas conductance and therefore water loss. However, such morphology would also limit the rate of diffusion of oxygen and CO_2

across the eggshell, potentially decreasing the chance of survival at high temperatures. Therefore, we examined the role eggshell traits played in embryonic survival. We found that successfully hatched individuals had 18.4 (±2.60, ±95% CI) pores per cm^2 of eggshell compared to 16.1 (±1.50, ±95% CI) pores per cm^2 of eggshell in embryos that died during development (LMM: t = 2.36, d.f. = 75, P = 0.021, random effect (SD) = 2.83). However, no difference in eggshell thickness between embryos that lived and died were observed (LMM: t = 1.37, d.f. = 79, P = 0.175, random effect (SD) = 0.0044).

While it is clear that eggshell characteristics can influence the physiology, development, and survival of avian embryos, little is known about their potential for plasticity and ability to facilitate anticipatory maternal effects. Therefore, we examined how eggshell pore density and thickness are influenced by thermal conditions experienced by the mother. We found that among mothers exposed to the high heat treatment as adults, those that also underwent the mild heat conditioning treatment as juveniles produced eggs with 4.26 (±3.96, ±95% CI) more pores/cm^2 compared to those that did not (LMM: MH-CH: t = 2.20, d.f. = 29, P = 0.036, random effect (SD) = 2.38). We also saw that these mothers exposed to the heat treatments at both timepoints (Mild-High) produced eggs with 5.38 (±4.36, ±95% CI) more pores/cm^2 than Control-Control mothers as well (LMM: t = 2.52, d.f. = 29, P = 0.018, random effect (SD) = 2.38), while we saw no differences in eggshell thickness due to experimental treatments (Table S5). Interestingly, this increase in pore density did not correlate with increased water loss, even at high incubation temperatures. This pattern may be due in part to the lower heart rates of embryos from Mild-High mothers at high temperatures, which paired with the increased number of pores potentially signals a reduced demand for oxygen rather than limitations in gas conductance.

In zebra finches, an incubation temperature of just one degree Celsius above optimum has been shown to decrease both hatching success and lean body mass in male embryos (Wada et al., 2015; Wada et al., 2018). As expected, here we saw that embryos incubated at the high temperature were 5.42 times more likely to die than those incubated at the control temperature (GLMM: $\beta = 1.69$, 95% CI = [0.384, 3.12], $z = 2.45$, $P = 0.014$, random effect (SD) = 0.245). We presumed that the lower water loss, reduced heart rates, and higher pore density shown by embryos produced by heat-exposed mothers would promote increased survival at high temperatures, however this was not the case. Among embryos exposed to the high incubation temperature, maternal treatment had no effect on survival (Fig. 6) (Table S6). There are a few possible reasons for this non-significance. It may be that any maternal effects potentially beneficial for embryos were not strong enough to counteract the negative effects of the high incubation temperature on embryo survival. Another possibility is that the potential benefits or constraints of those physiological changes are only seen on a different scale of observation and/or do not present themselves until later on in life. Early life cellular level damage can have effects persisting into adulthood. For example, high incubation temperatures and faster embryonic development rates were shown to reduce the length of telomeric DNA sequences in Japanese quail (Stier et al., 2020). This reduction in telomere length persisted into adulthood, and such shortening is associated with reduced longevity in many species (Heidinger et al., 2012). Therefore, while effects such as longer development times may not improve short term-survival, it may help avoid increases in damage at high incubation temperature and improve survival outcomes in adulthood.

While we found no effect of maternal treatment on embryo survival at a high incubation temperature, we did observe a maternal effect at control temperatures. At the control incubation

temperature, embryos from mothers that experienced the mild heat as juveniles were 3.05 times more likely to die if the mothers were also exposed to the high heat stress as adults (Mild-High) (Fig. 6) (GLM: MC-MH: $\beta = 1.12$, 95% CI = [0.084, 2.19], $z = 2.10$, $P = 0.036$). This difference is largely due to the drop in survival late in the incubation period around pipping, where the Mild-High group had a 29.2% drop in overall survival, compared to a 11.6%, 10.8% and 9.6% decrease for the other three groups (Mild-Cont, Cont-High, Cont-Cont respectively). Such a detrimental effect on offspring fitness may be due to the mismatch between maternal and offspring environment. Potentially, maternal exposure to elevated ambient temperatures during both development and adulthood was a strong signal that her offspring will also experience increased ambient temperatures, strongly necessitating a phenotypic change to maintain fitness (Sheriff and Love, 2013). However, at control incubation temperatures, the embryo's environment did not match the maternal conditions which induced the phenotypic change, resulting in decreased survival (Marshall and Uller, 2007).

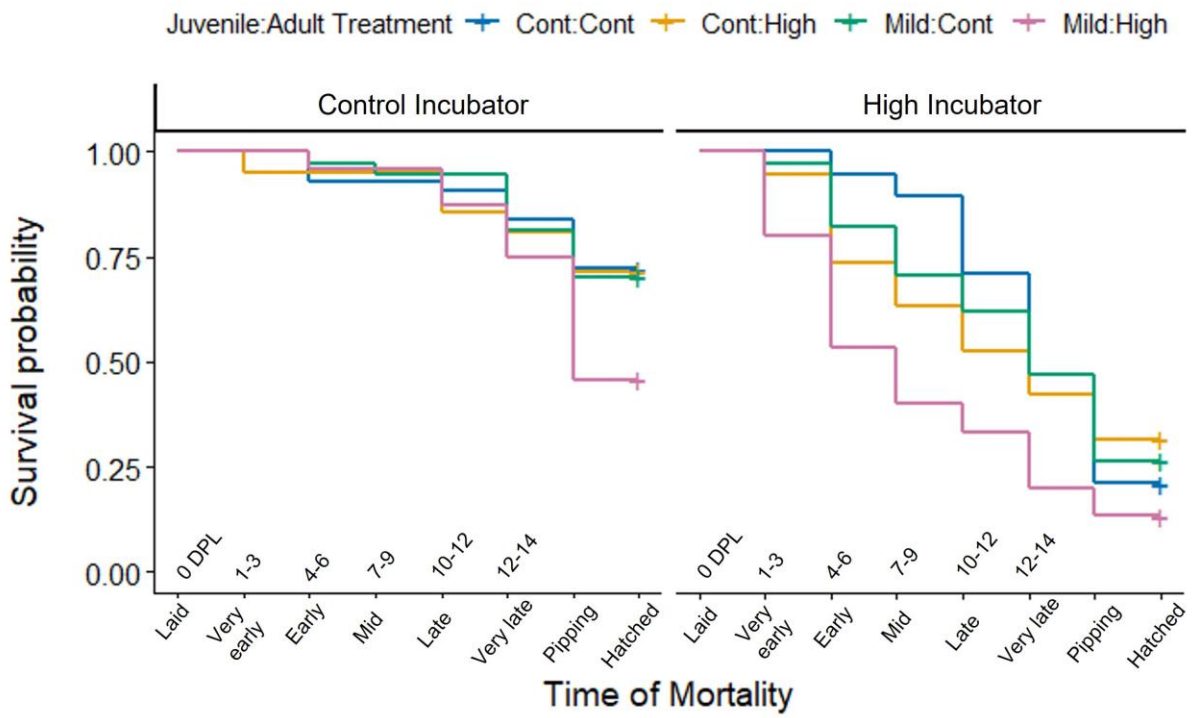

Fig. 6. Embryonic survival in relation to maternal treatment at each incubation temperature. Survival probabilities of embryos at different points in embryonic development, grouped by the respective thermal treatments received by their mothers. The x-axis denotes the estimated developmental stage (and the corresponding number of days) at which mortality of embryos occurred or if they successfully hatched, with "DPL" corresponding to "days post-laid". The left half of the graph shows embryonic survival at the control incubation temperature (37.2 °C) while the right shows survival at the high incubation temperature (38.5 °C). In the legend, the first word denotes the treatment the mothers received as juveniles (control or mild heat) and the second word denotes which treatment they received as adults (control or high heat).

While interpreting the adaptive significance of maternal effects is complex due to their highly context dependent nature, our study demonstrates the importance of designing experiments that incorporate several different time points and magnitudes of stressor exposure. Many of the effects we observed were the result of maternal exposure to a mild and prolonged heat conditioning as juveniles and its effect on the phenotype of embryos that developed at the high incubation temperature. Such changes in offspring phenotype as the result of an interaction between "matching" maternal and offspring environment may signal the presence of anticipatory maternal effects, however, further research is required. Our findings support previous studies which have demonstrated that the juvenile period is an important ontogenetic window for

inducing not only within-generation plasticity, but also intergenerational plasticity. While the plasticity of the mother's phenotype was not the focus of this study, the maternal treatment protocol utilized in this study has been previously demonstrated to induce within-generation plasticity in zebra finches (Costantini et al., 2012). Specifically, the juvenile mild heat treatment induced conditioning hormesis, preventing finches from suffering increased blood plasma oxidative damage after exposure to the high heat stressor as adults. The ability to induce adaptive maternal effects may interact with or even depend on the mother's own ability to induce within-generation plasticity. As such, the mild heat conditioning of mothers may also induce conditioning hormesis in the offspring, dubbed "transhormesis" by (Costantini, 2022). However, we hesitate to claim the presence of a transhormetic effect in this study, as we did not measure physiological costs and saw no beneficial effects of maternal treatment on survival at high temperatures. Nonetheless, the duration of the mild heat conditioning likely played a significant role in its ability to generate plastic responses, as the 28-day treatment period represents a significant portion of the developmental period in zebra finches (~90 days from hatching to sexual maturity) (Zann, 1996). Similar effects have previously been demonstrated, as adult sheepshead minnows exposed to 24 °C and 34 °C for 30 days produced offspring with increased growth at the temperature their parents were subjected to (Salinas and Munch, 2012). However, this parental effect was not seen when the parents were exposed to the temperatures for 7 days rather than 30 days. Findings such as these support the idea that environmental cues of a longer duration are more likely to induce anticipatory maternal effects, as they present a strong indicator that there will be a high correlation between the maternal and offspring environment.

Interestingly, the high heat stressor treatment as adults did not induce a selfish maternal effect, or any effect independent of its interaction with the juvenile mild heat treatment. This lack

of effect is surprising, as the time just prior to and during a reproductive bout is considered another critical window for inducing maternal effects (Donelson et al., 2018). Stressful environments can often create a trade-off between self-maintenance and the current reproductive bout for a mother resulting in selfish maternal effects (Marshall and Uller, 2007). However, we observed no apparent detrimental effects of the maternal high heat stressor (independent of its interaction with the juvenile treatment) on the offspring or maternal phenotype (see Hoffman et al., 2018). Potentially, trade-offs were reduced in this study via plentiful resources, or the stressor was not strong enough or close enough to the reproductive bout to elicit a cost. Mothers had *ad lib* access to seed and water except when undergoing experimental treatments, potentially allowing them to increase consumption to negate detrimental effects of the stressor. Often a longer environmental cue near reproduction will result in a stronger intergenerational effect (Salinas and Munch, 2012; Donelson et al., 2018; Dupont et al., 2013; Ho and Burggren, 2012). If the high heat stressor mothers experienced as adults was a longer duration or closer to the reproductive bout, we may have observed a more pronounced effect. Another possibility is that we did not capture the phenotypic costs because we did not measure the affected traits, or because they were not present at the timescale at which we measured. Potentially, the cost of this stressor exposure for mothers may only be seen at a cellular level (see Costantini et al., 2012) or may only be seen later in life such as reduced longevity (Costantini et al., 2014). Similarly, we measured phenotypic traits in offspring only prenatally and at hatch, and an induced selfish maternal effect may not be apparent until later on in development. Traits have different capacities for plasticity and involvement in trade-offs, which underpins the importance of measuring multiple traits and factoring in life-history in future studies on maternal effects moving forward. (Angilletta, 2009).

Conclusions

Our results suggest that thermal conditions experienced by the mother early on in life can induce maternal effects that alter the phenotype and development of embryonic offspring. Such plasticity could play a key role in species that live in regions experiencing temperatures close to their upper thermal tolerance, which are thought to be at greater risk of diminished populations as a result of climate change. Our results also indicate that thermal conditions experienced by the mother, even very early on in life, can elicit a phenotypic change in her offspring prenatally. Such information is critical, as the embryos of many avian species develop near their upper thermal critical limits and have very little thermoregulatory capabilities of their own (Webb, 1987; Mortola, 2009). Even though avian parents can regulate egg temperature, their methods to prevent hyperthermia when ambient temperatures rise above the optimum incubation temperature often comes at a significant cost to themselves (Grant, 1982). As climate change causes increased temperatures worldwide as well as an increase in extreme temperature events, the importance of understanding the rescue capabilities of phenotypic plasticity for species grows (Chevin et al., 2010). Understanding physiological and morphological adjustments of embryos due to maternal effects could improve our ability to predict population responses to climate change and pinpoint species that will be most at risk. Future studies in maternal effects would do well to utilize multiple ecologically relevant stressor magnitudes and exposure time points, as well as measuring a suite of traits in offspring over the course of their life-history in varying environments.

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

Statement of authorship

AJH and HW designed the study, AJH, LD, and HW carried out the experiment and collected data, AJH and HW performed statistical analyses and prepared the manuscript.

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Competing Interests

The authors declare no competing interests.

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Supporting Information:

Tables S1-S6

	eggmass			**wtchange**			**d4hr**			**d10hr**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.11	1.06 – 1.16	**<0.001**	0.09	-0.14 – 0.32	0.442	186.07	155.66 – 216.48	**<0.001**	304.96	236.63 – 373.30	**<0.001**
jtreat [Mild]	0.00	-0.07 – 0.07	0.972	-0.03	-0.08 – 0.01	0.094	-9.77	-18.33 – -1.20	**0.026**	-7.07	-29.74 – 15.61	0.530
atreat [High]	0.01	-0.06 – 0.07	0.875	0.02	-0.01 – 0.06	0.158	-3.31	-11.70 – 5.08	0.438	-5.71	-29.21 – 17.78	0.624
layorder	0.00	0.00 – 0.00	**<0.001**									
jtreat [Mild] × atreat [High]	0.02	-0.07 – 0.11	0.715				11.81	-0.20 – 23.81	0.054	24.23	-9.16 – 57.62	0.149
inctreat [HIGH]				-0.06	-0.10 – -0.01	**0.020**	17.67	11.73 – 23.61	**<0.001**	-12.34	-40.93 – 16.24	0.395
d4hr				-0.00	-0.00 – 0.00	0.936						
d10hr				-0.00	-0.00 – -0.00	**0.007**						
jtreat [Mild] × inctreat [HIGH]				0.07	-0.00 – 0.14	0.056				7.68	-26.68 – 42.03	0.659
eggmass							22.35	-2.52 – 47.21	0.078	17.17	-37.33 – 71.67	0.534
atreat [High] × inctreat [HIGH]										2.75	-32.38 – 37.89	0.877
(jtreat [Mild] × atreat [High]) × inctreat [HIGH]										-75.33	-127.50 – -23.15	**0.005**
Random Effects												
σ^2	0.005606			0.003080			604.936871			1576.523960		
τ_{00}	0.009814_{FID}			0.000000_{FID}			0.177994_{FID}			24.844264_{FID}		
ICC	0.636442			0.000000			0.000294					
N	41_{FID}			27_{FID}			40_{FID}			37_{FID}		
Observations	496			53			271			187		

Supporting Table S1. The statistical output of *LMMs* used to analyze the effect of maternal and incubation treatment on initial egg mass (g) ("eggmass"), egg mass change during incubation (g) ("wtchange"), early development heart rate (bpm) ("d4hr"), and late development heart rate (bpm) ("d10hr").

	d10hr		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	239.18	170.01 – 308.34	**<0.001**
eggmass	17.17	-37.34 – 71.68	0.535
MHH	*Reference*		
CCC	65.79	32.53 – 99.04	**<0.001**
CCH	53.45	17.11 – 89.78	**0.004**
CHC	60.08	28.33 – 91.82	**<0.001**
CHH	50.49	18.44 – 82.53	**0.002**
MCC	58.72	27.64 – 89.80	**<0.001**
MCH	54.06	22.21 – 85.91	**0.001**
MHC	77.24	43.76 – 110.72	**<0.001**
Random Effects			
σ^2	1576.523960		
$\tau_{00\ FID}$	24.844264		
N_{FID}	37		
Observations	187		
Marginal R^2 / Conditional R^2	0.114 / NA		

Supporting Table S2. The statistical output of the *LMM* used to analyze the effect of maternal and incubation treatments on the heart rate of embryos late in development (bpm) ("d10hr") using a categorical treatment variable that represents the eight combinations of maternal juvenile, maternal adult, and incubation treatments.

	inctime			inctime			pec			pec		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	15.32	14.01 – 16.63	**<0.001**	14.35	13.00 – 15.69	**<0.001**	-0.00	-0.03 – 0.03	0.890	-0.01	-0.04 – 0.02	0.644
jtreat [Cont]	-0.97	-1.81 – -0.14	**0.024**				-0.00	-0.01 – 0.00	0.173			
inctreat [CONT]	0.06	-0.39 – 0.50	0.797				-0.00	-0.01 – 0.00	0.067			
atreat [Cont]	-0.13	-0.77 – 0.51	0.684	-0.13	-0.77 – 0.51	0.684	0.00	-0.00 – 0.00	0.845	0.00	-0.00 – 0.00	0.845
d10hr	-0.00	-0.01 – 0.00	0.078	-0.00	-0.01 – 0.00	0.079						
jtreat [Cont] × inctreat [CONT]	0.38	-0.25 – 1.01	0.228				0.00	-0.00 – 0.01	0.289			
juv inccont × cont				0.44	-0.01 – 0.89	0.056						
juv incmild × cont				1.03	0.31 – 1.75	**0.006**				0.00	-0.00 – 0.00	0.709
juv incmild × high				0.97	0.16 – 1.79	**0.020**				0.01	0.00 – 0.01	**0.045**
body							0.03	0.01 – 0.04	**<0.001**	0.03	0.01 – 0.04	**<0.001**
inctime							-0.00	-0.00 – 0.00	0.976	-0.00	-0.00 – 0.00	0.976
juv inccont × high										0.00	-0.00 – 0.01	0.677
Random Effects												
σ^2	0.274321			0.274321			0.000043			0.000043		
τ_{00}	0.517590 $_{FID}$			0.517590 $_{FID}$			0.000000 $_{FID}$			0.000000 $_{FID}$		
ICC	0.653596			0.653596			0.000000			0.000000		
N	28 $_{FID}$			28 $_{FID}$			27 $_{FID}$			27 $_{FID}$		
Observations	79			79			93			93		
Marginal R^2 / Conditional R^2	0.143 / 0.703			0.220 / 0.730			0.169 / 0.169			0.169 / 0.169		

Supporting Table S3. The statistical output of *LMMs* used to analyze the effect of maternal and incubation treatments on incubation duration (days) ("inctime"), and pectoralis mass of hatchlings (g) ("pec"). Two statistical models were used for analysis of each dependent variable, the first was an *LMM* with juvenile and incubation treatment as fixed factors, and a juvenile*incubation treatment interaction term. The second model was for post-hoc analysis and was done using a *LMM* which included a categorical treatment variable representing the four combinations of maternal juvenile and incubation treatment.

	body			heart			yolk		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.64	0.15 – 1.13	**0.011**	0.00	-0.00 – 0.00	0.238	-0.04	-0.08 – 0.01	0.113
jtreat [Mild]	0.02	-0.04 – 0.08	0.475	0.00	-0.00 – 0.00	0.369	0.00	-0.02 – 0.02	0.911
atreat [High]	0.04	-0.02 – 0.10	0.182	-0.00	-0.00 – 0.00	0.887	-0.01	-0.03 – 0.01	0.499
inctreat [HIGH]	-0.02	-0.12 – 0.09	0.742	-0.00	-0.00 – 0.00	0.222	0.01	-0.02 – 0.04	0.574
eggmass	0.51	0.33 – 0.68	**<0.001**						
inctime	-0.02	-0.05 – 0.00	0.084						
d10hr	-0.00	-0.00 – 0.00	0.070						
jtreat [Mild] × atreat [High]	-0.04	-0.13 – 0.05	0.369	-0.00	-0.00 – 0.00	0.753	0.01	-0.02 – 0.04	0.562
jtreat [Mild] × inctreat [HIGH]	0.00	-0.12 – 0.12	0.994	0.00	-0.00 – 0.00	0.433	-0.02	-0.05 – 0.02	0.394
atreat [High] × inctreat [HIGH]	-0.04	-0.17 – 0.08	0.487	0.00	-0.00 – 0.00	0.324	-0.01	-0.05 – 0.03	0.668
(jtreat [Mild] × atreat [High]) × inctreat [HIGH]	0.04	-0.17 – 0.26	0.677	-0.00	-0.00 – 0.00	0.799	-0.01	-0.08 – 0.05	0.667
body				0.01	0.00 – 0.01	**<0.001**	0.15	0.09 – 0.21	**<0.001**
Random Effects									
σ^2	0.005759			0.000001			0.000403		
τ_{00}	0.000000_{FID}			0.000000_{FID}			0.000134_{FID}		
ICC	0.000000			0.000000			0.249352		
N	28_{FID}			27_{FID}			27_{FID}		
Observations	78			87			73		
Marginal R^2 / Conditional R^2	0.386 / 0.386			0.221 / 0.221			0.262 / 0.446		

Supporting Table S4. The statistical output of *LMMs* used to analyze the effect of maternal and incubation treatment on hatchling total body mass (g)("body"), heart mass (g)("heart"), and residual yolk mass (g)("yolk").

	Poredens			avgthickness			Poredens			avgthickness			Poredens			avgthickness		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	-2.30	-31.13 – 26.52	0.874	0.02	-0.01 – 0.06	0.179	31.28	20.05 – 42.50	**<0.001**	0.04	0.03 – 0.06	**<0.001**	31.28	20.05 – 42.50	**<0.001**	0.04	0.03 – 0.06	**<0.001**
survival	34.69	5.44 – 63.94	**0.021**	0.02	-0.01 – 0.06	0.183												
eggmass	17.76	-7.48 – 43.01	0.165	0.03	-0.00 – 0.06	0.097	-10.10	-19.77 – -0.43	**0.041**	0.01	0.00 – 0.03	**0.049**	-10.10	-19.77 – -0.43	**0.041**	0.01	0.00 – 0.03	**0.049**
survival × eggmass	-31.74	-57.22 – -6.26	**0.015**	-0.02	-0.05 – 0.01	0.225												
jtreat [Control]							-4.26	-8.22 – -0.31	**0.036**	-0.00	-0.01 – 0.00	0.539						
atreat [Control]							-2.89	-6.77 – 0.99	0.138	-0.00	-0.01 – 0.00	0.312						
jtreat [Control] × atreat [Control]							1.78	-3.81 – 7.37	0.520	0.01	-0.00 – 0.01	0.161						
treatment [CC]													-5.38	-9.74 – -1.01	**0.017**	0.00	-0.01 – 0.01	0.725
treatment [CH]													-4.26	-8.22 – -0.31	**0.036**	-0.00	-0.01 – 0.00	0.539
treatment [MC]													-2.89	-6.77 – 0.99	0.138	-0.00	-0.01 – 0.00	0.312
Random Effects																		
σ^2	19.553254			0.000030			21.284377			0.000031			21.284377			0.000031		
τ_{00}	7.995612_{nestid}			0.000019_{nestid}			5.662509_{nestid}			0.000019_{nestid}			5.662509_{nestid}			0.000019_{nestid}		
ICC				0.389834						0.382644						0.382644		
N	33_{nestid}			33_{nestid}			33_{nestid}			33_{nestid}			33_{nestid}			33_{nestid}		
Observations	111			114			115			118			115			118		
Marginal R^2 / Conditional R^2	0.143 / NA			0.059 / 0.426			0.161 / NA			0.074 / 0.429			0.161 / NA			0.074 / 0.429		

Supporting Table S5. The statistical output of *LMMs* used to analyze the effect of eggshell pore density (pores/cm^2) ("Poredens") and average thickness (mm) ("avgthickness") on embryo survival across all samples, as well as the effects of maternal and incubation treatments on eggshell pore density and thickness. For analysis of the effects of maternal and incubation treatments on eggshell pore density and thickness, two statistical models were used for each dependent variable. The first models were *LMMs* with maternal juvenile and adult treatment as fixed factors, and a juvenile*adult treatment interaction term. The second models were used for post-hoc analysis and were done using a *LMM*s which included a categorical treatment variable representing the four combinations of maternal juvenile and adult treatment.

	Survival (both incubators)			Survival (control incubator)			Survival (high incubator)		
	survival			**survival**			**survival**		
Predictors	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*
(Intercept)	0.40	0.15 – 1.07	0.067	1.18	0.53 – 2.69	0.683	6.50	1.80 – 41.57	**0.014**
jtreat [Mild]	0.95	0.29 – 3.16	0.939						
atreat [High]	1.07	0.32 – 3.61	0.912						
inctreat [HIGH]	5.55	1.41 – 21.85	**0.014**						
jtreat [Mild] × atreat [High]	2.83	0.55 – 14.47	0.211						
jtreat [Mild] × inctreat [HIGH]	1.81	0.33 – 10.00	0.499						
atreat [High] × inctreat [HIGH]	1.20	0.21 – 6.70	0.836						
(jtreat [Mild] × atreat [High]) × inctreat [HIGH]	0.46	0.03 – 6.45	0.568						
treatment [MC]				0.33	0.11 – 0.92	**0.036**	0.58	0.08 – 2.71	0.521
treatment [CC]				0.34	0.09 – 1.14	0.087	0.33	0.04 – 1.76	0.225
treatment [CH]				0.36	0.12 – 1.03	0.060	0.43	0.06 – 1.97	0.319
Random Effects									
σ^2	3.289868								
τ_{00}	0.060145 $_{FID}$								
ICC	0.017954								
N	36 $_{FID}$								
Observations	231			125			106		
Marginal R^2 / Conditional R^2	0.243 / 0.256			0.045			0.017		

Supporting Table S6. The statistical output of *GLMs* used to analyze the effect of maternal and incubation treatments on embryo survival/hatch success. The first was a *GLMM* which included juvenile, adult, and incubation treatment as fixed effects, as well as the two- and three-way interaction terms. Two further *GLM*s were used for post-hoc analysis where the data was subset by incubation treatment (control or high) and included the maternal juvenile*adult categorical treatment variable as a fixed factor.

Chapter 7: General Conclusions

In this dissertation I demonstrate that a mild developmental stressor can have beneficial and potentially adaptive effects on the phenotype in zebra finches, and indirectly influence the phenotype of their offspring. However, these effects were context dependent in both predictable as well as unforeseen ways. As anticipated, the adaptiveness depended on a match between the early life and adult environment, as well as the response trait being evaluated. However, many of the observed effects were also dependent upon on the sex, the timing of the sampling, and even the tissue used to measure the response trait. The context dependent nature of our results highlights how beneficial effects of developmental stressors could be easily missed, and why developmental stressors were long seen as purely harmful to phenotype, rather than tailoring it to specific conditions.

The results found throughout the chapters of this dissertation provide strong support for the presence of adaptive developmental plasticity (predictive adaptive response) resulting from the early life mild heat conditioning, and generally support the environmental matching hypothesis (Gluckman et al., 2005; Monaghan, 2008). When measuring different traits, we often saw that the finches exposed to the mild heat conditioning as juveniles and the high heat stressor as adults (mild:high) had seemingly better performance outcomes than one of or both groups with “mismatched” juvenile and adult treatment (cont:high & mild:cont). These mild:high finches had faster wound healing rates and latency to lay eggs (Ch. 2), lower oxidative damage, (Ch. 3), higher antioxidant enzyme and heat-shock protein levels (Ch. 3 & Ch. 4), and higher rates of survival (Ch. 5). The only potentially negative outcome these mild:high finches exhibited was an increase in basal DNA damage, however, this seemingly did not translate into detrimental effects on other traits. Finches exposed to the mild heat conditioning (regardless of

the adult treatment) also had higher adult body masses (Ch. 5), and produced offspring with adjusted embryonic phenotypes that may increase fitness in a post-natal environment with high ambient temperatures (Ch. 6). Together they indicate that the mild heat conditioning during development induced an adaptive change in phenotype that made the finches more suited for an adult environment with elevated temperatures. However, did we demonstrate that the mild heat conditioning induced a conditioning hormetic effect specifically, avoiding the negative impacts of the high heat stressor as adults? That answer is more complicated. The presence of conditioning hormesis would be determined by comparing the groups exposed to the high heat stressor as adults, and examining whether those that were also exposed to the mild heat conditioning as juveniles had better performance outcomes than the juvenile controls (mild:high vs cont:high). Moreso, you would expect that these non-conditioned finches exposed to the high heat stressor as adults would exhibit worse performance outcomes compared to finches who were not exposed to the heat treatment at either time point (cont:high vs. cont:cont). We saw such a response pattern when looking at several traits, namely the latency to lay (Ch. 2), some antioxidant enzymes (Ch. 3), heat-shock proteins (Ch. 4), and perhaps the biggest piece of support, lipid oxidative damage (Ch 3). However, such patterns of conditioning hormesis were observed mostly in tissue-level measurements, and were nearly absent when looking at fitness-related traits at a higher biological level such as reproduction and survival. Part of the issue is that contrary to our predictions, many of the fitness-related traits we measured were not negatively impacted by exposure to the high heat stressor (comparing cont:cont vs. cont:high), which is necessary to truly test for conditioning hormetic effects in specific traits.

The lack of observed negative effects on traits resulting from exposure to the adult high heat stressor protocol (42 °C for 3 hours/day, for 3 consecutive days) raises the question of

whether it actually poses a significant stressor to the finches. The temperature of 42 °C is significantly higher than the upper critical temperature of the thermoneutral zone for the species, but still below the lethal temperature (Calder, 1964; Wojciechowski et al., 2021). Moreso, this specific high heat stressor protocol was chosen based on previous studies that demonstrated that it resulted in increased plasma oxidative damage and reduced survival in zebra finches (Costantini et al., 2012, 2014). While we did see that exposure to this high heat stressor resulted in increased cellular lipid damage (measured via 4-HNE) in male finch livers (Ch. 3) and egg laying latency (Ch. 2), they were essentially the only results that could be definitively interpreted as a detrimental effect of the protocol. The high heat protocol also had no observed negative effects on the offspring of exposed females (Ch. 6), implying the mothers did not face a trade-off between self-maintenance and the current reproductive bout that induced a selfish maternal effect. The question then remains, was this high heat protocol just not that stressful to our finches, or did we miss the effects? Based on early results (Ch. 2), we speculated that we did not observe negative effects of the high heat protocol on wound healing and some female reproductive parameters potentially due to a shift in life-history strategy. We believed that the high heat protocol may have triggered females to invest more resources into the current reproductive bout, at the expense of self-maintenance and longevity. However, contrary to our speculation, we later saw that these females did not exhibit a reduction in survival (Ch. 5). One explanation is that finches were able to avoid trade-offs and negative impacts on traits when exposed to the high heat protocol through increased food intake. Finches had *ad lib* access to seed and water except when undergoing the thermal treatments, which may have allowed them to match an increase in energetic costs associated with exposure to the stressor, negating its detrimental effects. It is also possible that we simply did not detect the phenotypic costs because

we did not measure the affected traits and/or measure traits at the correct time-point. This seems somewhat unlikely, as we measured key fitness related traits at multiple biological levels, and often at multiple timepoints. Perhaps in support of the idea that the high heat protocol did not pose a significant challenge to our finches, recent studies have shown that zebra finches are generally able to avoid decreases in body mass and increases in CORT when experiencing similar temperatures during heat waves in the wild (Cooper et al., 2019, Cooper et al., 2020). Regardless, these ideas underline how researchers examining developmental plasticity should take significant care when selecting the traits to be measured and the environmental manipulations with regards to ecological realism.

In further support of the concepts of predictive adaptive responses and environmental matching, there were costs associated with the phenotypic change resulting from the juvenile mild heat conditioning with regards to several traits, such as wound healing (Ch. 2) and survival (Ch.5). However, these costs only presented when there was a "mismatch" between the early and later life environments, represented by conditioned finches that were not exposed to the high heat stressor later as adults (mild:cont). Such costs of developmental stressors are potentially less likely than adult stressors to be offset through increased resource availability, as their persisting effects are not only achieved through early life resource reallocation, but through a divergence away from the default developmental trajectory that is irreversible (Natelson et al., 1988; Spencer and MacDougall-Shackleton, 2011). This idea that the phenotypic adjustments resulting from stressful developmental environments become maladaptive when the adult environment is good is an extension of an environmental matching model called the "thrifty phenotype" (Monaghan, 2008). The extended model attempts to explain why humans that developed in poor nutritional conditions experience obesity and metabolic dysregulation later in life under nutrient

rich conditions, and this has been adopted by biologists in the study of developmental plasticity (Hales and Barker, 2001; Fernandez-Twinn and Ozanne, 2006). Studies investigating the presence of environmental matching/predictive adaptive responses have seen mixed results, although results that support the predictions of such models have been demonstrated in birds, including zebra finches (Saastamoinen et al., 2010; Noguera et al., 2015).

So why has there been such a lack of support for environmental matching-mismatching models? The answer may lie in the species being studied. As previously discussed, one of the core principles of the adaptive potential of developmental stressors is that that the environmental cue eliciting the change in phenotype is variable and somewhat predictive of future conditions (Gluckman et al., 2005; Bateson, 2001; Bateson et al., 2014). Therefore, populations that experience conditions that do not vary considerably over the lifetime of the individual, or experience conditions that are extremely unpredictable and increase the chances of a mismatch, are not likely to exhibit adaptive developmental plasticity (DeWitt et al., 1998, Scheiner and Holt, 2012). These conditions depend on the life history of the species. Environmental conditions may vary at a pace that is too unpredictable for one species but be considered predictable for another species, depending on their lifespan and the time it takes to reach sexual maturity. This could explain why studies supporting the environmental matching hypothesis were done using species that develop quickly such as the zebra finch, which reaches sexual maturity at around 3 months post-hatch (Zann, 1996). Potentially, another reason that the environmental matching hypothesis is lacking in empirical again goes back to how developmental stressors were often studied. As discussed previously, the persisting effects of developmental stressors were often evaluated in a single non-stressful adult environment (Uller et al., 2013; Groothius and Taborsky, 2015; Taborsky, 2017). In this way, many studies were unknowingly observing the detrimental

effects of the developmental stressor when there was an environmental mismatch, and potentially missing the benefits when there is a match between the early and later life environments. As further demonstrated by this dissertation, experiments with full-factorial designs are essential when examining developmental plasticity. Ideally, there would be more than two levels for each treatment as to create experimental designs with more ecological realism and potentially capture non-linear reaction norms, although we realize this poses significant logistical hurdles (Herczeg et al., 2016; Taborsky, 2017).

While the research in this dissertation demonstrates maladaptive effects developmental stress when there was an environmental mismatch, the mechanisms regulating these costs are still somewhat unclear. We saw that exposure to the mild heat conditioning early in life resulted in persisting change in several physiological traits involved in the stress response such as antioxidant enzymes (Ch. 3), heat-shock proteins (Ch. 4), and corticosterone secretion (Ch. 5). In some instances, we saw associations between traits at the same biological levels of organization that supported the presence and mediation of trade-offs. For example, in Chapter 3 we saw that higher liver SOD-1 antioxidant enzyme levels were associated with lower liver oxidative damage, but higher testes SOD-1 and SOD-2 antioxidant enzyme levels were associated with higher levels of liver oxidative damage. However, when looking at relationships between traits at the cellular level and those at a higher biological level (e.g., reproduction, survival), we often saw that the relationships were not good explanatory markers for the variation seen in our higher-level biological traits resulting from our experimental treatments. For example, DNA damage is thought to be one of the underlying causes of ageing and is often correlated with survival, and yet in our experiment we saw no associations between genomic DNA damage in erythrocytes and survival in female finches (Ch. 5). An obvious explanation is that we did not

see associations between such traits due to the influence of mechanisms we did not measure, such as DNA repair rates (which may be a better predictor of lifespan than damage itself), lysosomal function, cellular signaling, or mitochondrial biology (Whitehead and Grigliatti, 1993; Hyun et al., 2008; Robert and Bronikiwski, 2010).

Developmental plasticity in mitochondrial biology is particularly interesting as another potential mechanism mediating the effects of thermal treatments seen in this dissertation. Mitochondrial biology can act as a link between glucocorticoids, cellular damage, reproduction, and longevity (Finkel and Holbrook, 2000; Zhang et al., 2016; Hood et al., 2018; Casagrande et al., 2020). Changes in mitochondrial biology can be induced by stressors in order to meet energetic demands that can result in long lasting changes in aerobic cellular metabolism (Youle et al., 2012; Pichaud et al., 2019; Stier et al., 2022). The function and cellular density of mitochondria are two traits which are subject stress-induced plasticity, and there is evidence in birds that an individual's mitochondrial biology can exhibit developmental programming (similar to the HPA-axis) that may be adaptive (Gyllenhammer et al., 2020; Cossin-Sevrin et al., 2022; Stier et al., 2022). However, if there is mismatch between energetic demand and mitochondrial phenotype, detrimental outcomes may occur. For example, overexpression of the PGC1α transcription factor in mice cardiac tissue led to excessive mitochondrial biogenesis and death (Finck and Kelly, 2007). Therefore, it is possible that in our study the mild heat conditioning resulted in a persistent change in mitochondrial abundance in anticipation of a future with frequent stress, but was maladaptive when the adult environment was benign (mild:cont). Specifically, exposure to the mild heat conditioning during development may have increased energetic demands, CORT, or ROS levels, all of which have been shown to trigger mitochondrial biogenesis (Weber et al., 2002; Manoli et al., 2007; Scarpulla et al., 2012; Picard et al., 2018).

The early life mild heat conditioning in our study may have also resulted in an adjustment in mitochondrial efficiency. Mitochondrial efficiency is the plastic coupling of ATP synthesis with oxygen consumption during oxidative phosphorylation (Nicholls and Ferguson, 2002; Divakaruni and Brand, 2011; Helle et al., 2012; Galli et al., 2016). A reduction of this coupling increases proton leakage and decreases mitochondrial efficiency, which can have beneficial effects under some circumstances through the reduction of ROS production (Nicholls and Ferguson, 2002; Halliwell and Gutteridge, 2015; Salin et al, 2015). Furthermore, in zebra finches specifically it has been demonstrated that heat exposure during pre- and post-natal development can result in potentially adaptive alteration in mitochondrial efficiency and leak (Udino et al., 2021; Pacheco-Fuentes et al., 2023). It is possible that in our study the mild heat conditioning resulted in a persistent increase in mitochondrial uncoupling and leak (potentially through elevations in CORT) to reduce basal ROS production (Casagrande et al., 2020). Such a phenotypic change could be adaptive if the adult environment is expected to have frequent heat stressors that would increase ROS further to a damaging degree. However, such an increase in uncoupling could have been maladaptive in the non-stressful adult environment, by reducing basal ROS production to a level that disrupts essential ROS mediated signaling pathways (discussed in Ch. 6) (Sies, 2014; Ristow and Schmeisser, 2011). Such a reduction in ROS production could also explain the higher masses in conditioned finches, as experimentally reducing mitochondrial ROS levels improved growth in wild yellow-legged gull (*Larus michahellis*) chicks (Velando et al., 2019). In light of all this and their well-documented role in mediating the life-history of individuals, we propose investigations into mitochondrial biology should be a key avenue of investigation as a mechanism underlying adaptive developmental plasticity in future studies (Speakman et al., 2015; Hood et al., 2018, Heine and Hood, 2020).

In closing, my dissertation contributes to the understanding of adaptive developmental plasticity, particularly in endothermic vertebrates. It is clear that mild stressors during development can have direct and influential effects on phenotype, as well as indirect effects on the subsequent generation, and therefore may play a large role in the persistence and evolution of populations. It is my hope that this research provides key considerations and jumping off points for future studies, or at least makes someone think "that's pretty interesting!".

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