Effects of Periodic Cooling During Incubation on Lifelong Physiology in Zebra Finches

by

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Abstract

Developmental stress can have long term consequences on phenotypes. In birds, incubation temperature is critically deterministic for a range of traits. When parents leave the nest to forage, developing embryos can be exposed to cooling events which can have long term effects on offspring development. To investigate the immediate and long-term consequences of periodic cooling on offspring development and physiology, we exposed zebra finch eggs to periodic cooling events 5 times a day for 30 minutes throughout the incubation period. Additionally, we incubated eggs at a constant optimum control temperature of 37.4°C and at a constant low temperature of 36.4°C. During embryonic development, we measured embryonic heart rates, embryonic mass change, duration of incubation, and survival to hatch. Post-hatch, we measured offspring growth as well as assessed adrenocortical function at baseline levels and in response to an acute stressor. We saw significant increases in embryonic heart rate early in development in periodically cooled and low temperature eggs while we only observed significant decreases in heart rates later development in the low treatment. Additionally, we saw increases in incubation duration in cooled and low temperature eggs, transient decreases in post-hatch growth in the cooling treatment, and significant differences between controls and the low constant temperature treatment in terms of the integrated adrenocortical response to an acute stressor. There were also differences in the stress induced adrenocortical response between and within treatments with age. These results indicate that periodic cooling during incubation significantly alters developmental metabolism, which has consequences on post-hatch growth and the ability to respond to a repeated acute stressor. Furthermore, the effect of constant low versus fluctuating incubation temperatures has different biological effects on both embryonic and post-hatch growth and physiology, which must be disentangled from one another to properly evaluate how organisms respond to temperature stress during early development.

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Table of Contents

List of Figures

Chapter 1: Periodic Cooling During Incubation Alters Embryonic Heart Rate and Development

Introduction

Organisms display a great deal of reversible acclimation and plasticity during development that allows them to partially reconcile the effects of a suboptimal environment (Beaman *et al.* 2016). The degree of plasticity and habituation displayed during early life stages is partly dependent on the intensity of a stressor experienced during development. For oviparous species, incubation temperature is critical for proper embryonic development and has lifelong consequences on phenotype (Durant *et al* 2012; Wada *et al.* 2015). The response to changes in temperature during the embryonic period has been well studied in non-avian reptiles (e.g., Angilleta *et al.* 2000; Warner and Shine 2011; Wyneken and Lolavar 2017; Liang *et al.* 2015; While *et al.* 2018; Noble *et al.* 2018). However, considerably less is known about the relationship between development, thermal environment, and plasticity in avian species (Booth 2018; Du and Shine 2015).

Unlike non-avian reptiles, birds have a relatively narrow range of temperatures at which embryos can properly develop (Du and Shine 2015). The temperatures embryos experience are largely mediated and buffered by parental incubation behavior, and maintenance of optimum nest temperatures represents a large energetic investment (Conway and Martin 2000; Ardia *et al.* 2009; Ardia *et al.* 2010). When resources are scarce, this can come at a cost to parents because they must leave the nest to maintain their own energetic needs thus exposing developing embryos to temperature fluctuations causing eggs to cool and rewarm (Conway and Martin 2000; Zann and Rosetto 1991). Most studies on avian embryos have examined embryonic development at constant temperatures (ex. Kolackova *et al.* 2014; Durant *et al.* 2012; Eiby and Booth 2009). In natural systems though, avian embryos likely experience a great deal of temperature fluctuations (Zann

and Rosetto 1999). This can have lifelong physiological and morphological consequences, and may also play a role in determining the degree of phenotypic variation due to plasticity organisms are able to display (Beaman *et al.* 2016; Crino and Breuner 2015; Burness *et al.* 2013). Fluctuating incubation conditions are known to alter growth as well as reduce growth efficiency, potentially due to differential allocation of resources during development (Olson *et al.* 2006; Olson *et al.* 2008). Yet, very little is known about how cooling events during incubation influence metabolism, growth, and survival. Additionally, few studies have attempted to disentangle the effects of changes in mean temperature from changes in the amount of temperature variation specifically in relation to avian embryonic development. This is critical as changes in mean temperature and changes in variance in temperature can have differential and intertwined biological effects (Bozinovic *et al.* 2011; Lawson *et al*. 2015).

Measuring embryonic heart rate represents an effective and non-invasive way to asses embryonic development and metabolism in oviparous species (Hall and Warner 2018; Sheldon *et al*. 2018). Modulation of embryonic heart rates can indicate developmental rate in relation to incubation temperature and may also represent a way for some embryos to thermoregulate (Ar and Tazawa; Du *et al.* 2013; Du and Shine 2015). While commonly used in studies on embryos of non-avian reptiles (ex. Hall and Warner 2018; Du *et al.* 2013; Hulbert *et al.* 2017), the use of embryonic heart rates via a Buddy digital heart rate monitor has only recently been experimentally validated for birds (Sheldon *et al.* 2018). The Buddy system allows evaluation of the effects of incubation temperature on avian embryonic development and can be used to generate large data sets of heart rates repeated over the course of development. In order to statistically assess such a large data set, one can use a multilevel regression, also known as a random regression, to accurately capture the large amount of within and between individual variation present (Dingemanse and Dochterman 2013). This statistical technique allows accurate

assessment of plasticity and has applications in detecting functional tradeoffs (Westneat *et al.* 2011; Careau and Wilson 2017).

This study had two goals: first, we aimed to assess the embryonic heart rate data with a random regression, a novel statistical approach in this system, in order to fully capture the amount of individual variation caused by different incubation treatments. Second, we aimed to determine the effects of fluctuating incubation temperatures on embryonic development in the model bird species *Taeniopygia guttata* (zebra finch) to test the hypotheses that 1) periodic cooling during incubation alters embryonic metabolism 2) this effect would be less detrimental than a constant low incubation temperature with the same average temperature, and 3) cooling would improve hatchling condition and survival as it more closely mimics natural environmental conditions. Zebra finches are ideal to study the effects of fluctuating incubation temperature because their embryonic development is well characterized under a range of conditions and they readily breed in captivity. To test the aforementioned hypotheses, we exposed developing embryos to one of three incubation regimes: a constant 37.4°C, a periodic cooling treatment, and a constant 36.4°C hereafter referred to as Constant, Periodic, and Low, respectively. We then assessed embryonic heart rate with a random regression, as well as evaluated length of incubation, survival to hatch, and mass change over the course of the incubation period.

Materials and Methods

Experimental Setup and Animal Husbandry

Male (n=32) and female (n=32) zebra finches from a captive breeding colony housed at Auburn University were paired to produce eggs from April 2018 to December 2018. Breeding pairs (n=32) were placed in individual cages and supplied with an external cardboard nest box (19.5 x 14.5 x 14.5 cm) and nesting materials of shredded paper and irradiated hay. Fertile eggs were only produced by a subset of these nests (n=25). All birds were provided *ad libitum* access

to seed (Kaytee Supreme (Finch), Chilton, WI), water, grit, and cuttlefish bone. Each pair was given a tablespoon of egg food mixture daily, as well as a weekly spinach and vitamin supplement. To stimulate breeding behavior, pairs were spritzed with water daily until egg laying commenced.

At the onset of egg laying for each nest, eggs were labeled with a non-toxic fine tipped marker. The initial egg from each clutch was left in the nest to ensure nest integrity, and all subsequent eggs were removed and replaced with either infertile or sham clay eggs. Nests were checked daily between 10 am and 12 pm for newly laid eggs. Removed eggs were randomly assigned to one of three Brinsea Octagon 20 Advance EX incubators (Brinsea Products Inc. Titusville, FL, USA). Incubator treatments included a Control incubator that held a constant temperature of 37.4°C, a Periodic incubator that utilized a programmable switch to cool down five times a day for 30-minute intervals and held a constant 37.4°C overnight, and a Low incubator that held a constant temperature of 36.4°C (**Figure 1**). The Low incubator temperature was the experimentally determined average temperature of the Periodic incubator. Humidity in each incubator was held at a constant 55%. Eggs were randomly assigned to an incubator balancing both laying order and nest of origin.

At approximately 30% development (Day 4 or 5 pre-hatch), eggs were candled to check for fertilization. At approximately 90% development (Day 13 or 14), eggs were moved to one of two hatchers that held constant temperatures of 37.4°C for Control and Periodic eggs or 36.4°C for Low eggs (**Figure 2**). This was to ensure that temperature fluctuations were only experienced during development and not post-hatch. Hatchers were set to 60% humidity and were checked three times a day for hatched or pipping individuals. Upon hatching, individuals were individually marked by unique feather cut patterns and were returned to nests. Hatchlings were cross-fostered and assigned a rearing nest so that clutch size was between 3-5 individuals for

each nest. When the oldest nestling was 5 days post hatch (dph), cages were switched to 2 tablespoons of daily egg food to reduce nestling competition. Parents were separated from offspring at nutritional independence (45 dph) at which point individuals no longer received daily egg food. Individuals were visually sex- separated between 55-60 dph. All procedures and protocols were approved by the Auburn University's Institutional Animal Care and Use Committee (Protocol #2018-3274).

Embryonic Heart Rates

Embryonic heart rates (HR) were taken at two time points, ~30% and ~80%, over the course of development using a Buddy digital heart rate monitor. To confirm that heart rates were taken at the same approximate time during development across treatments, the day heart rates were taken was divided by average length of incubation for each treatment (e.g. days 4 and 10 for Control; days 5 and 11 for Low and Periodic). Eggs in the Control treatment had their heart rates taken on pre-hatch day 4 and 11, approximately 28% and 77% development, while those in the Periodic and Low treatments had heart rates taken on day 5 and 12. This corresponded to 32.7% and 78.59% development in the Low treatment and 33% and 80.07% development in the Periodic group. To measure HR, individual eggs were removed from incubators and quickly candled to ensure fertility and survival. Heart rates were measured between 07:30 and 09:00 before temperature fluctuations began in the Periodic incubator and all incubators had held constant temperatures overnight. The egg was then placed in the Buddy system at room temperature, and the heart rate was recorded every 5 seconds for 65 seconds. Eggs were then returned to their respective incubator. HRs were measured in a haphazardly random fashion with regards to incubator assignment, and a minimum of 5 minutes passed before an incubator was opened again to measure another egg. Embryonic mass was measured three times over the course

of embryonic development; at laying before being placed in an incubator, at ~80% development after HR measures had been taken, and at hatch.

Statistical Analyses

All statistical analyses were performed in R version 3.5.2 (http://www.R-project.org/). Models were run using the lme4 package (http://cran.us.r-project.org/web/packages/lme4) unless otherwise specified. P values were retrieved using the lmerTest package (https://cran.r-project.org/web/packages/lmerTest). Variables were determined signficicant if $p<0.05$ and variables with p-values >0.1 were removed from models in a stepwise fashion.

The effect of incubator treatment on embryonic survival was determined using a binomial generalized linear mixed effects model with lay mass as a covariate and genetic nest as a random effect. Initial egg mass and change in egg mass over the duration of incubation were analyzed using separate but identical linear mixed effects models with a random effect of egg identity nested within genetic nest. Analyses at ~80% mass and mass at hatch included a fixed effect covariate of lay mass. These models were run using the nlme package (https://cran.r-project.org/web/packages/nlme/). Relative development time in days was analyzed using a linear model including treatment and mass at laying. However, mass at laying was removed due to non-significance. Models for change in mass and development time were only ran for embryos that survived to hatch.

Embryonic heart rates were analyzed using random regression with correlated random slope and intercepts (n=208). Separate but identical models were used for heart rates taken at ~30% development and ~80% development and given a correlated random slope and random intercept. A random effect of individual nested within treatment in regard to time was specified to indicate that slopes and intercepts varied both between treatments and between individuals within treatments. Furthermore, all data were minimum or left centered by time so that the first

measure taken for each egg was represented by 0. Due to a large amount of missing data where the Buddy system was not able to detect heart rates, sampling intervals between individuals and over time were unequal in their distribution. This was accounted for statistically by including an average time of measurement covariate for each individual in each model, which was also left centered (Plewis 1989; but see Dingmanse and Dochtermann 2013). To confirm that the random regression with correlated random slopes and random intercepts was the best fit model, we ran an ANOVA of a basic linear model, a model with uncorrelated random slopes and intercepts, and a model with correlated random slopes and intercepts. At both ~30% and ~80% development, the correlated model had the lowest AIC score. Autocorrelation in the data was assessed using the acf function in R and was determined acceptable as per Westneat *et al. (*2011).

Results

Survival, Mass Change, and Incubation Duration

There was no effect of incubation treatment on survival (p_{Low}=0.8734; p_{Per}=0.1297) nor on mass at hatch (p_{Low}=0.3091; p_{Per}=0.2644). However, at laying eggs in the Periodic treatment were initially 0.047 ± 0.0164 (mean ± se) grams or 4% lighter than those in the Control treatment (p=0.0051). As incubator assignment was randomized in regard to both genetic nest and laying order, this difference was due to random chance and is well within the range for natural variation in egg mass at laying within and between clutches. There was a significant effect of the Periodic treatment on egg mass at ~80% development (p=0.0218). Eggs in the Periodic treatment weighed 0.053 ±0.023 grams (mean± se) or 4.90% lighter than those in the Control group and 0.063 ± 0.022 grams or 5.9% lighter than those in the Low group (**Figure 3**). While there was no significant effect of lay mass on mass at ~80% or at hatch, lay mass was kept in all statistical models due to the initial difference between treatment groups. There was a significant effect of incubation treatment on the total length of development in both the Low and Periodic treatments. Eggs in the

Low incubator took 1.06. ±0.0416 days longer to hatch (p<2e-16), and those in the Periodic incubator took 0.7770 ± 0. 0.0388 days longer to hatch (p<2e-16) than eggs in the control incubator. Incubation duration of the Low and Periodic treatments was equivalent (**Figure 4**).

Embryonic Heart Rates

At ~30% development, both the Low and Periodic treatment significantly increased embryonic heart rate. The Low treatment increased heart rate by 13.77 ± 3.80 beats per minute (bpm), and the Periodic treatment increased heart rate by 17.71 ± 3.47 bpm (p_{Low}=0.00038; p_{Per}=9.48e-07). Additionally, time out of the incubator (p=< 2e-16) as well as the average time of measurement (p=0.000801) were significant covariates in the model. These results are shown in **Fig. 5A**. At ~80% development, only the Low treatment had a significant effect on embryonic heart rate when compared eggs in the Control treatment (**Fig. 5B**). Embryos in the Low treatment had 15.34 ± 4.78 slower hear beats than embryos in the Control treatment (p=0.0017). Time out of the incubator was the only significant covariate in the model (p< 2e-16).

Discussion

We tested how periodic cooling during incubation alters embryonic development and embryonic heart rates in zebra finches. We found that incubation treatment did not influence embryonic survival or mass at hatch. However, there was a significant effect of the Periodic treatment on mass at ~80 % development in embryos that survived to hatching. The effect of the Periodic incubation treatment on embryonic heart rate changed over the course of development. Heart rates in the Periodic and Low groups were higher early at ~30% development but at ~80% development only the Low group was affected, and had lower heart rates. There was also a significant increase in the duration of development in both the Low and Periodic treatments. This supported our hypotheses that fluctuating incubation temperatures alter embryonic development and that this effect was different from that observed for the Low treatment, which was the average

temperature of the Periodic treatment. We did not see support for temperature fluctuations improving hatchling condition in term of mass or survival in comparison to a low constant temperature.

The length of the incubation period in the Periodic treatment (~15.0 days) and the Low treatment (~15.3 days) was greater in comparison to that of the Control treatment (~14.2 days) (shown in **Fig. 3**). This increase in incubation length is likely due to changes in metabolism related to incubation conditions. Fluctuating and low incubation temperatures are known to increase the energetic demands placed on embryos, which lengthens the duration of incubation (Olson *et al*. 2006; Olson *et al*. 2008). We did not observe any immediate consequences of incubation treatment on hatch mass or survival in the present study, which is similar to previous work in a range of oviparous species including zebra finches, wood ducks, blue tits and *Anolis* lizards (Wada *et al.* 2015; Carter *et al.* 2014; Nord and Nilsson 2014; Tiatragul *et al*. 2017). However, we did see differences in egg mass at ~80% development. The fact that eggs in the Periodic treatment were lighter at ~80% of the embryonic development indicates water loss was greater due to cooling and rewarming. It is also possible individuals reared under Periodic conditions devoted more energy to acclimating to temperature changes, and that the effects of incubation temperature alters allocation of energy without changes in body mass or survival. For instance, fluctuating incubation temperatures have been demonstrated to alter the strucutral composition, amount of residual yolk, and embryonic growth in unhatched zebra finches (Olson *et al*. 2006; Olson *et al.* 2008). Low incubation temperature altered total energy expenditure in wood ducks, which negatively impacted physiological performance at hatch (Durant *et al*. 2011) Additionally, increases in the duration of incubation are associated with decreased immunocompetence. In wood ducks, individuals reared in constant low incubation temperatures showed decreased swelling responses to phytohemattagluttnin and decreased antibody production to sheep red blood cells (Durant *et al*.

2012). Tree swallows from cooled nests also showed decreased innate immunity (Ardia *et al.* 2010). Furthermore, longer incubations have been shown to decrease post hatch survival in zebra finches (Wada *et al.* 2015). This indicates that the effects of developmental stress associated with low and fluctuating temperatures may only be observed in later life stages.

There are as of yet very few actual studies assessing the long-term effects of fluctuating incubation temperatures on offspring phenotype in birds, and the underlying physiological mechanisms that determine responses to low constant temperatures versus temperature fluctuations are not well understood. In a study of Japanese quail, individuals exposed to fluctuating incubation temperatures did not differ in their adult metabolic rates when compared to individuals from constant low and control temperatures (Ben-Ezra and Burness 2017). This is in contrast to Nord and Nilsson (2011) which found permanent effects of low constant incubation temperatures on metabolism, and Wada *et al*. (2015) which saw transient sex-specific effects of constant low incubation temperatures on metabolism and physiology. Furthermore, there is evidence that cooling during post-hatch development alters growth and the adrenocortical response to an acute stressor (Lynn and Kern 2016), and is discussed in Chapter 2. These differences in long-term effects of incubation and developmental environment represent a promising avenue of study for testing understanding how developmental environments shape lifelong physiology and for testing predictive adaptive responses.

Our analysis of embryonic heart rate represents an application of a novel statistical approach for heart rate measures that strengthens an already useful tool for assessing developmental metabolism and rate in avian species (Sheldon *et al.* 2018). While the Buddy digital heart rate monitor is commonly used in studies interested in embryonic development, its use necessitates some level of disturbance which may confound experimental effects as the embryonic heart rates of both birds and non-avian reptiles are extremely sensitive to changes in the thermal

environment. Unless heart rates are taken in a temperature-controlled chamber it may be difficult to disentangle the effect of an experimental treatment from moving eggs out of an incubator or nest to measure their heart rate, as this in and of itself represents a cooling event and embryonic stressor that can decrease heart rate. Additionally, while Sheldon *et al.* (2018) reports that repeated single reliable heart rate measures can be used to evaluate embryonic heart rates, single timepoint or maximum measures may upwardly bias estimates of effect (Careau and Wilson 2017). There was a relatively high degree of variability in the number of heart rate measurements each individual had for both ~30% and ~80% development as the Buddy system can fail to give readings if an embryo moves while its being measured (Sheldon *et al.* 2018). The random regression with a left centered fixed effect covariate of the average amount of time an embryo was measured for helped us account for the large amount of within and between individual variation between individuals at both ~30% and ~80% development without potentially overestimating experimental effects (Careau and Wilson 2017; Dingemanse and Doctermann 2013). This technique also allowed us to effectively estimate the effect of treatment on heart rate while accounting for autocorrelation associated with the logistics of taking repeated measures of embryonic heart rates of eggs removed incubators or nests.

Overall, our study found that embryonic heart rates were increased in both the Low and Periodic treatments at ~30% development (**Fig.5A**). At ~80% development, the Low treatment had lower heart rates while the Periodic treatment did not differ from the Control (**Fig. 5B**). All treatments increased their relative heart rates over the course of incubation. While the present study did not test for a mechanism of how these changes may be occurring, our results do support the idea that avian embryos have the capacity to physiologically and behaviorally acclimate to their environment thus displaying some degree of acclimation and reversible plasticity in regard to incubation temperature (Du and Shine 2015). The transient effect of

incubation conditions on embryonic heart rate in the Periodic treatment could be due to changes in the expression of the somatotrophic axis which is thought to mediate developmental plasticity related to metabolism in a range of species (Danzter and Swanson 2011). However, the effects observed are likely influenced by a host of physiological, behavioral, and biochemical parameters (Du and Shine 2105). This plasticity may also come at a cost to organisms later in life if it is not indicative of future conditions (Monaghan 2008; Beaman *et al.* 2016). Conversely, under consistently stressful conditions, represented by the Low treatment, embryos were unable to acclimate to their environment as their heart rates always differed from those in the Control. This may indicate a permanent change to their physiological phenotype. Constant low incubation temperatures have been shown to negatively impact performance for a range of physiological traits as well as diminish overall condition at hatch and into adult hood in several species (Ardia *et al.* 2010; Ben-Ezra and Burness 2017; Wada *et al.* 2015).

Differences in the effects of Periodic and Low treatments on heart rate illustrate that there can be dissimilar biological effects in regard to changes in mean temperature versus variation in temperature (Bozinovic *et al.* 2011; Lawson *et al.* 2015). This is well documented in the incubation of embryos of many non-avian reptiles, especially those displaying temperature dependent sex determination (ex. Warner and Shine 2011). This emphasizes the importance of assessing how embryos respond to thermal fluctuations. It is possible that organisms are somewhat robust to the effects of fluctuating incubation temperatures as nest temperatures vary greatly between and within species under natural conditions (Reyna and Burggren 2017; Durant *et al.* 2012; Zann and Rossetto 1991). Furthermore, it is important to recognize that physiological responses, growth, and survival of embryos differ between species as well as with the magnitude, duration, and form of temperature fluctuations (i.e. spikes, dip, or both) (Reyna and Burggren 2017; Du and Shine 2015).

In summary, we saw significant increases in incubation length in both the Low and Periodic treatments but only saw differences in Periodic heart rates early in development. This suggests a mechanism of physiological plasticity related to periodic cooling during the incubation period not displayed in response to constant low temperatures. Eggs in the Low treatment had lower heart rates at ~80% development showing that constant suboptimal conditions have differential effects on embryo physiology than when temperatures fluctuate, which may mimic temperature regimes that embryos experience in natural settings. We saw no difference in mass at hatch or survival related to incubation treatment but did see a transient effect on change in egg mass in the Periodic treatment. We have also demonstrated that random regressions represent an appropriate and useful statistical technique for analyzing embryonic heart rate measures taken of avian embryos exposed to different incubation temperatures. This method allows researchers to account for missing data points in large data sets of repeated measures, and reduce statistical bias introduced by using single timepoint or maximum measurements. This will greatly improve future studies utilizing heart rate measures to assess embryonic development.

Chapter 2: Effects of Periodic Cooling During Incubation on Post-hatch Growth and the Adrenocortical Responses in Zebra Finches

Introduction

Developmental stress can have lasting effects on organism phenotype (Monaghan 2008). This is usually thought of being solely negative but there is strong evidence that, depending on the magnitude of the stress experienced, some stressors can induce positive phenotypes (Crino and Breuner 2015; Hoffman *et al.* 2018). Developmental conditions also determine the capacity for reversible acclimation and plasticity displayed later in life (Beaman *et al.* 2016; Burness *et al.* 2013). The consequences of developmental environment on growth and physiological traits are therefore of critical importance to informing our understanding of organisms' capacity to appropriately respond to future stressors. Furthermore, if developmental stress changes the capacity to habituate to stressors, this may represent a reduced ability for the individual to adjust to new environmental conditions. Developmental stress consequently may influence whether a changing environment becomes a chronic stressor or something an individual can acclimatize to.

In birds, incubation temperature is known to be strongly deterministic for both embryonic development and survival (described in Chapter 1), as well as for post-hatch phenotypes, growth, and survival (Ardia *et al*. 2010; Burness *et al.* 2013; Wada *et al.* 2015; Bernsten and Bech 2016). Despite this, there have only been a few studies which examined life-long effects of incubation temperature and those largely have been conducted using constant incubation temperatures (see Wada *et al.* 2015). However, it is well documented that incubation temperatures in natural settings vary with ambient temperature and parental behavior. During reproduction, parents experience a tradeoff between self-maintenance and current reproductive success. In poor environments incubating females must leave nests more often to maintain their own energetic need exposing embryos to more frequent fluctuations during development (Zann and Rosetto 1991; Conway *et*

al. 2000; Carter *et al.* 2014). Moreover, one predicted consequence of global climate change is a greater degree of climatic instability meaning more frequent and dramatic shifts in ambient temperatures (Lawson *et al.* 2015). Parental incubation behavior and offspring responses to thermal fluctuations may therefore be indicative of how organisms will potentially respond to climate change and is in essential need of study (Huey *et al.* 2012). Changing mean temperatures can also have different biological effects than changing variation in temperatures (ex. Warner and Shine 2011; Ben-Ezra and Burness 2017). It is critical to understand the effects of both mean temperature changes and changes in temperature variation to understand and predict how organisms will respond to climate change.

Fluctuating incubation temperatures under laboratory conditions can mimic what embryos experience in natural settings. Since nest cooling occurs naturally and there is evidence that females in the wild already do not incubate optimally for embryonic development, embryos may be able to buffer themselves against temperature fluctuations to some extent, despite experiencing lower overall average nest temperatures, thereby reducing the energetic demands placed on parents (Ardia *et al.* 2010). This could potentially belay any long-term detriments associated with low incubation temperatures. For instance, Japanese quail raised under fluctuating incubation conditions had lower metabolic rates than those raised under constant low temperature conditions even though both experienced the same average temperature (Ben-Ezra and Burness 2017). We aimed to test the hypothesis that fluctuating incubation temperatures affect lifelong growth and physiology differently than constant low temperatures using the altricial songbird *T. guttata.* We measured birds' ability to elicit an adrenocortical response (e.g. corticosterone levels) to an acute stressor, as well as post-natal growth in body mass and skeletal size via tarsus measurements over the course of their post-natal development until they reached sexual maturity (~90 days post hatch). Based on previous work done in zebra finches, we predicted post-hatch growth (i.e. mass and

tarsus length) would not be affected by incubation treatment but there would be a delay in growth in the Periodic and Low treatments (Wada *et al.* 2015). We also predicted that the adrenocortical response would be elevated in both the Low and Periodic treatments with Periodic treatment displaying an intermediate phenotype between the Control and Low groups in terms of both growth and secretion of corticosterone (CORT) in response to an acute stressor.

Materials and Methods

The same experimental design and animal husbandry protocols were used as those previously described in this thesis. A total of 84 individuals were used in this experiment, although only 78 survived to sexual maturity.

Post Hatch Growth

Hatchling mass to the nearest mg was taken immediately post-hatching (day 0) before individuals were transferred to foster nests. Mass was also taken at 2, 5, 10, 16, 30, 40, 60, and 90 days post hatch (dph) (**Fig. 6**). For these days, mass was taken to the nearest .01g. Measurements of tarsus length were taken on 2, 5, 10, 30, and 60 dph. For 2, 5, and 10 dph, tarsus length was measured via digital photographs taken with a stationary, mounted Samsung S6 cellphone camera. Tarsi length were quantified using ImageJ with a 10 millimeter scale in each photo (Schneider *et al.* 2012). For 30 and 60 dph, tarsi were measured using calipers. All photos, Image J analyses, and caliper measurements were taken by one individual. All measurements of growth were taken before daily egg food was given.

Blood Measures

Blood samples were taken from all individuals three times over the course of the experiment at average nest age 16, 40, and 90 dph (**see Fig. 6**). These timepoints were chosen to sample individuals before fledging (16 dph), around nutritional independence (40 dph), and after they had reached sexual maturity (90 dph). Blood was collected via brachial venipuncture using

26-guage needles and into heparinized microhematocrit tubes. For each age, baseline and stress induced samples were collected. Baseline samples were taken following published procedures and were all completed within 3 minutes of entering the room (Wada *et al.* 2009; Wada *et al*. 2015). Following baseline sampling, individuals were placed in opaque paper bags for 30 minutes to induce a stress response at which point they were bled again. Samples were taken between 08:00h and 10:00h. After collection, blood samples were refrigerated until centrifugation in order to separate plasma from red blood cells. Plasma was aliquoted out into two tubes for further analyses. All samples were frozen within 2h of collection.

Corticosterone Quantification

Plasma corticosterone in baseline and post-capture and handling samples was quantified using enzyme-linked immunosorbent assay (Enzo Life Sciences, cat # ADI-901-097). This kit has been validated for zebra finch plasma previously (Wada *et al.* 2009). Recently, another validation assay showed that plasma dilution of 1:20 with 2.5% steroid displacement buffer offers a good low-dilution alternative to 1:40 dilution with 1.5% steroid displacement buffer (Wada; Unpublished data). Thus, the former condition was used in this study. All samples from each individual were run on the same plate with all treatment groups represented in each plate. Within each plate, all samples were haphazardously randomized. Intra- and inter- plate variation was 2.13% and 18.94%, respectively.

Statistical Analyses

Post hatch mass and tarsus growth were analyzed using linear mixed effects models with an interaction between treatment and age (in days), as well as a random effect of bird ID nested within rearing nest. Initially, a covariate of sex was included in the models but was removed due to insignificance. Where the age by treatment interaction was significant, analyses were broken down by age with rearing nest as random effect.

For baseline and stress-induced corticosterone levels at each age, the integrated response was calculated as the area under of the curve between 0 min of disturbance and stress induced CORT levels, assuming corticosterone levels stay constant between 0 min and 3 minutes of capture. This value represents the total amount of corticosterone released during the 30-minute capture restraint protocol. Baseline, stress induced, and integrated responses were analyzed as repeated measures across 3 ages as separate linear mixed effect models with an interaction between treatment and age as well as with an individual identity as a random effect. Due to the interaction between treatment and age, baseline, stress induced, and integrated CORT levels for each day were analyzed using linear models. Linear models for each for baseline, stress induced, and changes in integrated CORT levels over time were also analyzed within each treatment group.

Results

Post-hatch Growth

There was no overall significant effect of treatment on body mass (M_b). However, the effect of the Periodic treatment at 2 dph was marginally nonsignificant (p=0.0582) as birds were 0.13± 0.066 grams lighter than those in the Control group representing an 8.7% decrease in M_b. There were significant treatment by age interactions in the Periodic treatment at 5, 10, and 16 dph. On 5 dph, birds in the Periodic treatment were 0.59 ± 0.19 grams or 16.2% lighter than those in the Control treatment (p=0.0314). At 10 dph, birds incubated in the Periodic group were 1.0517± 0.43 grams or 16.02% lighter than those in Control group (p=0.0159). There was no individual effect of treatment on mass at 16 dph (p=0.113) as individuals were only 4% lighter, and there was no effect of treatment on M_b at any timepoint thereafter. At no time point over the course of the experiment did individuals in the Low group differ from those in the Control in regard to their M_b (**Fig 7**).

There was no overall significant effect of treatment on tarsus length. However, there was again a significant treatment by age interaction at multiple timepoints. Tarsus length did not differ among treatment groups at hatch (p_{Low}=0.931; p_{Per}=0.8832). On 2 dph, individuals in the Periodic treatment had 0.31± 0.14 millimeter (mm) or 6.41% shorter tarsi (p=0.0275) compared to Control nestlings. On 5 dph, nestlings in both the Low and Periodic groups had shorter tarsi (p_{Low}=0.0031; p_{Per}=0.00048) compared to nestlings in the Control group. Individuals in the Low treatment had 1.018± 0.331 mm or 12.99% shorter tarsi and those in the Periodic treatment had 1.4 ± 0.31 mm or 14.54% smaller tarsi compared to ones hatched from the Control incubator. On 10 dph, the Periodic treatment showed 1.14 ± 0.37 mm or 7.70% shorter tarsi than those in the Control treatment (p=0.00318). There was no effect of treatment on tarsus length at 30 or 60 dph, indicating that the effect of incubation temperature was temporary. (**Fig 8**).

Plasma CORT Levels

There was no significant interaction (treatment x age) for baseline measures. The interaction term was significant at both 40 and 90 dph for the Low treatment at stress induced levels. However, there was no effect of the Low treatment found on stress induced levels when analyzed by age group. There was no effect of incubation treatment on baseline or stress induced levels of corticosterone when analyzed as repeated or single timepoint measures for any treatment. Baseline and stress induced CORT levels over time are shown in **Figure 9** and **Figure 10,** respectively.

There was no change in baseline CORT levels within a treatment as nestlings got older. Within the Control, group stress induced CORT levels were 7.396 ± 2.248 ng/mL (p= 0.00169) lower at 40 dph and 7.177 ± 2.556 ng/mL (p= 0.00676) lower at 90 dph when compared to stress induced levels 16 dph. Stress induced levels between 40 and 90 dph individuals in the Control group did not differ in their stress induced CORT levels group, but males did have 3.901 ±1.483

ng/mL (p= 0.0108) lower CORT levels. In the Periodic group, stress induced CORT levels were 5.86760 ± 2.67180 ng/mL lower at 40 dph when compared to measures at 16 dph (p= 0.0309), but there was no difference between 40 and 90 dph or between 16 and 90 dph. Additionally, there was no effect of sex within the Periodic group. Stress induced CORT levels within the Low treatment did not change with age. These results, as well as within treatment changes in the integrated CORT response, are shown in **Figure 11**.

The integrated CORT response showed a significant age by treatment interaction for all ages for at least one treatment group, so analyses were broken down by age. At 16 dph, the Low treatment significantly decreased the integrated response by 62.669 ± 29.128 ng/(mL x min) (p=0.0039), and mass was a significant covariate in the model (p=0.0334). There was no effect of any treatment group on the integrated response at 40 dph although mass continued to be a significant covariate (p=0.0122). At 90 dph, the Low treatment significantly increased its integrated response by 50.146 ± 23.148 ng/(mL x min) (p =0.0254), and mass was again significant covariate (p= 0.00429) **(Fig 12).** Within the Control group, the integrated CORT response decreased by 85.87 ± 34.62 ng/(mL x min) at 40 dph and by 104.31± 39.36 ng/(mL x min) at 90 dph compared to 16 dph, but there was no difference between the response at 40 and 90 dph. In the Periodic group, the integrated response decreased by 81.682 ± 26.470 ng/(mL x min) at 40 dph (p= 0.00239), and by 58.759 ± 27.932 ng/(mL x min) at 90 dph (p=0.03695), but the magnitude of response at 40 and 90 dph was not different. Males in the Periodic group had 40.020 ± 16.130 ng/(mL x min) higher integrated CORT responses than females. In contrast to the Control and Periodic groups, the integrated CORT response for individuals in the Low treatment did not change over time (shown in **Fig. 11**).

Discussion

Cooling during incubation altered post-hatch growth in terms of both body mass and tarsus length, as well as influenced the adrenocortical response to an acute stressor. We found transient effects of experimental treatment on both mass and tarsus length with the Periodic treatment having a stronger effect on nestling growth than the Low treatment. This was indicated by the fact that the Periodic treatment suppressed both body mass and tarsus growth for the majority of the nestling period while the Low treatment only reduced tarsus growth at one age. Baseline and stress induced CORT levels did not differ with age between treatments but generally all treatments had decreased stress induced CORT levels over time (**Figs. 9** and **10)**. When the effect of treatment was analyzed at each day, the Low treatment had a significantly lower integrated CORT response at 16 dph compared the Control group, and levels stayed constant thereon. At 90 dph, the Low treatment had a higher integrated adrenocortical response in comparison to the Control treatment (**Fig 12**).

Skeletal growth in early life decreased in both the Low and Periodic treatments. However, this effect was not present at hatch and disappeared before birds fledged. This is similar to the effect of incubation temperature treatment seen on M_b wherein M_b in the Periodic treatment was lower in early life, but the effect was transient, possibly due to catch-up growth during the late stages of the nestling period. Catch up growth has been shown to occur in zebra finches exposed to nutritional stressors during development and had lifelong consequences on resting metabolic rate (Criscoulo *et al.* 2008). While this may be directly related to effect of incubation treatment, it is also possible that the reasons behind the transient decreases in mass and skeletal growth observed are due to Periodic individuals being less competitive in getting food from parents during the nestling period soon after hatch. Foster parents in the experiment had *ad libitum* access to food and this potentially allowed individuals to offset the negative effects of incubation temperature on

M_b. Decreased food availability is known to slow and alter growth but how it interacts with incubation temperature is as of yet unstudied (Lepcyzk and Karasov 2000; Crino and Bruner 2015). Additionally, we saw no sex-specific effects of incubation treatment on growth or body mass. This this is contrary to some previous work but there is mounting evidence that suboptimal incubation temperatures cause sex-specific physiological differences even when morphological differences are not present (Gurley *et al* 2018; Bernsten and Bech 2016; Wada *et al.* 2015; Ben-Ezra & Burness 2017). It is possible that sex-specific effects may not manifest in the traits measured in this study, and furthermore if sex-specific effects on growth are transient as previously reported (see Wada *et al.*2015), the timing of measurements of this study may have simply missed the windows in which they could be observed.

Wada *et al.* (2015) observed that zebra finches incubated at constant high and low temperatures had transiently lower M_b in early life, but saw no age by treatment interactions. We also saw no effect of constantly low incubation temperature on body mass, but saw strong effects on growth in the Periodic treatment. These two studies together point to differential effects of constant and fluctuating temperatures on growth, even when the average temperature is the same. Further study of long-term or latent effects of this depression in growth is needed. The overall pattern of delayed post-hatch growth seen here and in previous work may represent a common response in zebra finches to suboptimal incubation conditions regardless of whether temperatures are higher or lower (Wada *et al* 2015). In precocial Japanese quail, fluctuating incubation temperatures altered body mass in adulthood but not growth rate (Ben-Ezra and Burness 2017). Similarly, precocial wood ducks incubated under fluctuating incubation temperatures did not differ in their early life growth post hatch (Carter *et al.* 2014; Durant *et al.* 2012). These differences in post hatch growth rate are likely due to differential allocation of resources to structural growth versus tissue and organ development during the embryonic period known to

occur during stressful incubation conditions (Olson *et al.* 2006; Olson *et al.* 2008; Durant *et al.* 2011). Responses to fluctuating incubation temperatures may differ between precocial and altricial species as precocial species are more developed at hatching than altricial species. Precocial species may preferentially allocate more energy during development to structural growth in response to stressful conditions as they must be independently mobile at hatch (Wada 2018). This may have latent effects on post-hatch grow or adult condition, and in turn could potentially impact the development of endocrine systems and responses, especially in regard to future stress tolerance and the development of thermoregulatory capacity (Durant *et al.* 2013; Ben-Ezra and Burness 2017; Wada *et al* 2018).

Baseline glucocorticoid levels were not affected by treatment at any time point post-hatch nor did baseline CORT levels change within treatments between age groups. This is similar to previous work done in zebra finches and eastern bluebirds, both altricial species, that showed no difference in baseline CORT levels in comparison to controls (Wada *et al.* 2015; Lynn & Kern 2016). This is in contrast to stress-induced and integrated CORT responses which were affected by incubation treatment. While stressed-induced CORT levels did not differ between treatment groups at any point, the Control group displayed reduced stress induced levels after 16 dph. This indicates that birds in the Control group habituated to capture and handling stress over time. In contrast, the Periodic treatment decreased stress induced CORT level between 16 and 40 dph but stress-induced levels did not differ between 16 and 90 dph, possibly representing a diminished ability to habituate to capture and handling. The Low treatment's stress-induced CORT levels did not change with age, indicating an inability to habituate to capture and handling. Habituation to a stressor, as seen in the Control group, is critical for animals to avoid accumulation of the "wear-and-tear" associated with chronic elevation of corticosterone levels as put forth in the Reactive Scope Model (Romero *et al.* 2009). In the context of the Reactive Scope Model, the

inability to habituate to stressor indicates that low constant incubation temperatures decreased the range of reactive homeostasis, which is an organism's capacity to deal with changes in the external environment while maintaining normal homeostatic functions. This means individuals in the Low treatment could either be operating more closely to their upper homeostatic limits or are else more easily pushed into homeostatic overload where CORT itself starts to harm the body. Inability to habituate to a stressor could lead to lifelong higher CORT and supports previous work that lower incubation temperatures elevate the adrenocortical response, which has been shown to decrease immunocompetence, growth, and survival (Sapolsky *et al*. 2000; Wada *et al.* 2015; Jimeno *et al*. 2018). Our findings support the idea that developmental stress can influence the ability to deal with acute and repeated stressors throughout life, possibly via altering physiological and biochemical mechanisms regulating to HPA axis responsiveness such as glucocorticoid receptor density (Love *et al.* 2013).

Stressors experienced during early life can also alter development of the hypothalamic-pituitary-adrenal (HPA) axis (Lynn *et al.* 2013; Lynn and Kern 2016). The HPA axis mediates numerous physiological processes but in terms of adrenocortical response, it is the primary regulator of CORT secretion thus allowing organisms to respond to changes in the environment by increasing the energy available, in the form of glucose, to cells and tissues (Sapolsky *et al.* 2000). The development of the HPA axis is therefore critical for organisms to be able to respond appropriately to stressors and potential danger. Integrated CORT was lower in the Low treatment at 16 dph compared to the Control but was not different between the Periodic and Control treatments. At 90 dph the Low group had significantly increased integrated CORT responses when compared to Control. This difference in the Low treatment suggests that low constant incubation temperatures caused a delay in the development of the HPA axis as responsiveness in early life was depressed (**Fig. 12**). Interestingly, integrated CORT responses within the Periodic

group decreased from 16 to 40 dph but increased from 40 to 90 dph. While this was not statistically significant, it may suggest that the Periodic group represents an intermediate state of HPA axis function between the Control and Low treatment, and that temperature fluctuations buffered the effects of low incubation temperatures on the development of the HPA axis. Additionally, the HPA axis plays a critical role in tempering the magnitude of the adrenocortical response with prior exposure and future work may provide a mechanistic explanation for the differences seen in the capacity for habituation between treatments.

Overall, fluctuating incubation temperatures were not detrimental to growth or HPA axis function. However, we observed sex-specific differences in the integrated response within the Periodic treatment suggesting that sexes may differ in how they are affected by temperature fluctuations. Female zebra finches raised under constant low incubation temperatures had transient increases in their adrenocortical response (Wada *et al.* 2015). Female zebra finch embryos raised under constant high temperatures also showed larger pectoralis muscles and less residual yolk than females incubated under control temperatures, a difference not seen in males raised under the same conditions (Gurley *et al.* 2018). Additionally, other types of early life stressors have also been shown to have sex-specific consequences related to the CORT response. Early life food restriction and exogenous CORT exposure in song sparrows had differential effects on the production of sex steroids in males and females, as well as influenced sex-specific growth and metabolic rates (Schmidt *et al*. 2012; Schmidt *et al*. 2014). This suggests that there are differences in the underlying responses to early life stress between the sexes, although this may differ with the type of stressor experienced.

In summary, we saw transient effects of incubation treatment on skeletal growth and body mass. Additionally, while we saw overall decreases in the CORT response with age there was a significant increase in integrated CORT levels in the Low treatment in comparison to the Control

group. The Control group was able to habituate to the acute stressor of capture and restraint decreasing its adrenocortical response over time. Individuals from the Periodic treatment were also able to accomplish this, just at a reduced scale. Periodic cooling during incubation therefore represents an intermediate state in terms of the CORT response to an acute stressor with age as CORT levels never differed from either the Control or Low group for any measure of CORT, supporting our hypothesis that periodic cooling does alter lifelong growth and physiology. Whether these differences are beneficial remains to be seen. As expected, zebra finches exposed to cooling did display transient differences in growth but surprisingly we did not see this in individuals incubated under constant low conditions. We did not see strong evidence that low temperatures or periodic cooling alter the adrenocortical response but, instead our results suggest that low constant temperatures may hinder the ability to regulate the response over time. Lastly, physiological phenotypes induced by fluctuating incubation temperatures may represent an intermediate state between constant low and optimal incubation temperatures that allows offspring to grow relatively normally under suboptimal environmental conditions reducing the cost of reproduction to parents while still maintaining reproductive success. Future work should aim to evaluate whether phenotypes induced by variable incubation temperatures are adaptive and under what conditions in order to test the environmental matching hypothesis (see Monaghan 2008), wherein if environments in early life are indicative of those experienced later on, fitness is increased.

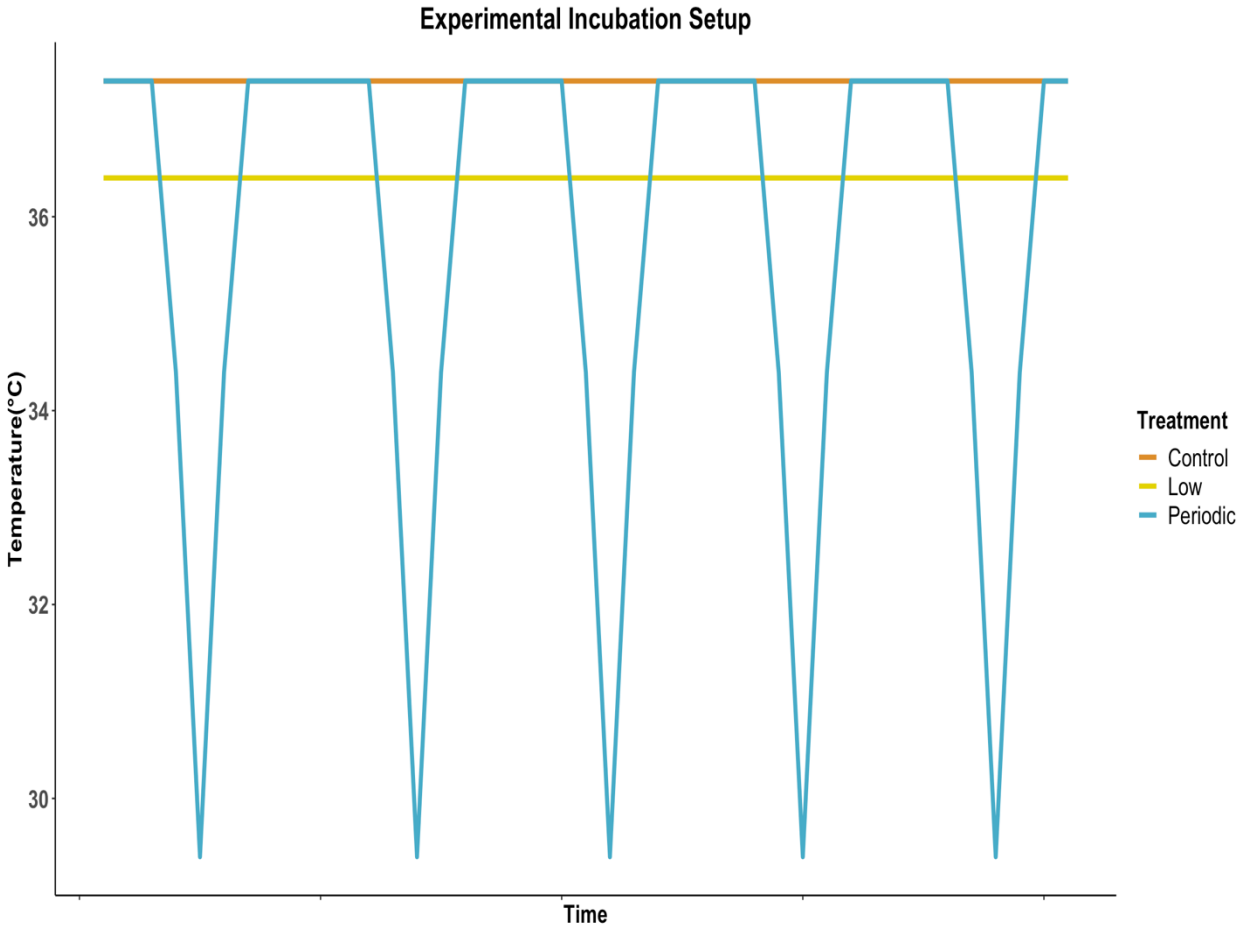

Figure 1. Graphical representation of incubation regimes. Control (in orange) represents constant 37. 4°C and Periodic (in blue) represents incubator that cooled down 5 times a day for 30 minutes and held a constant 37.4°C overnight. The Low (in yellow) represents constant 36.4°C which was the determined average temperature of the Periodic incubator.

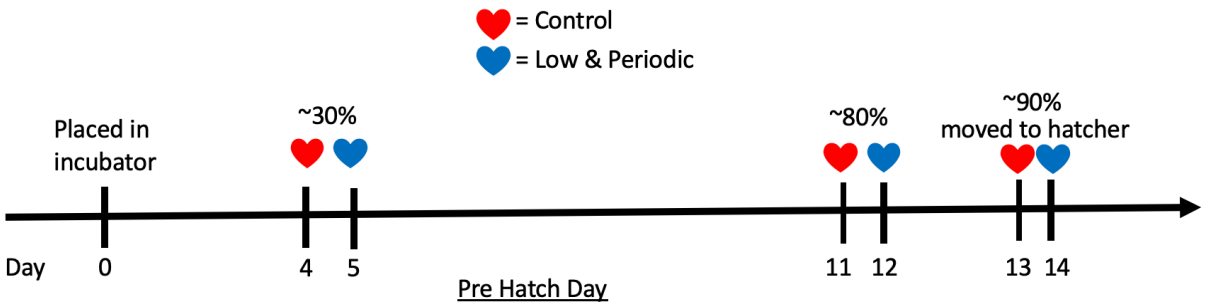

Figure 2. Experimental timeline for embryonic development and heart rate measurements. Heart rates were taken at ~30% and ~80% development and all eggs were moved to hatchers at ~90% development. Red hearts indicate days Control heart rates were taken or eggs were moved. Blue hearts represent days Low or Periodic heart rates were taken or eggs were moved.

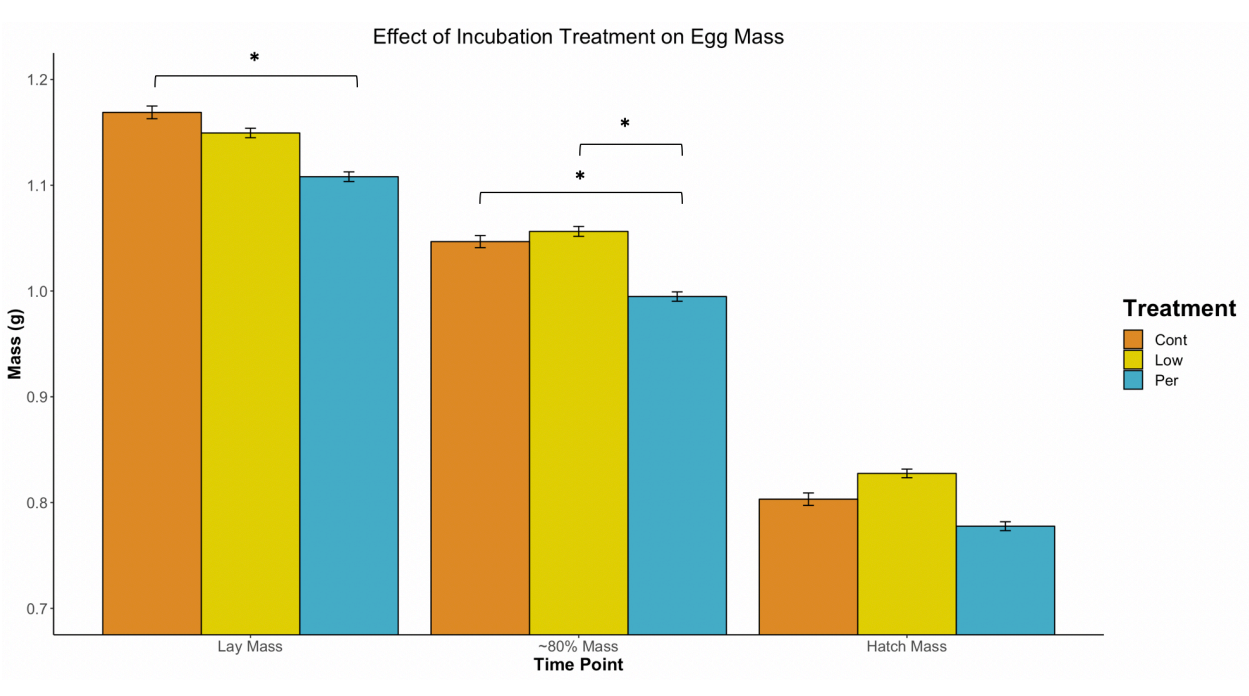

Figure 3. Change in egg mass from laying to hatch. Significance is indicated by *.

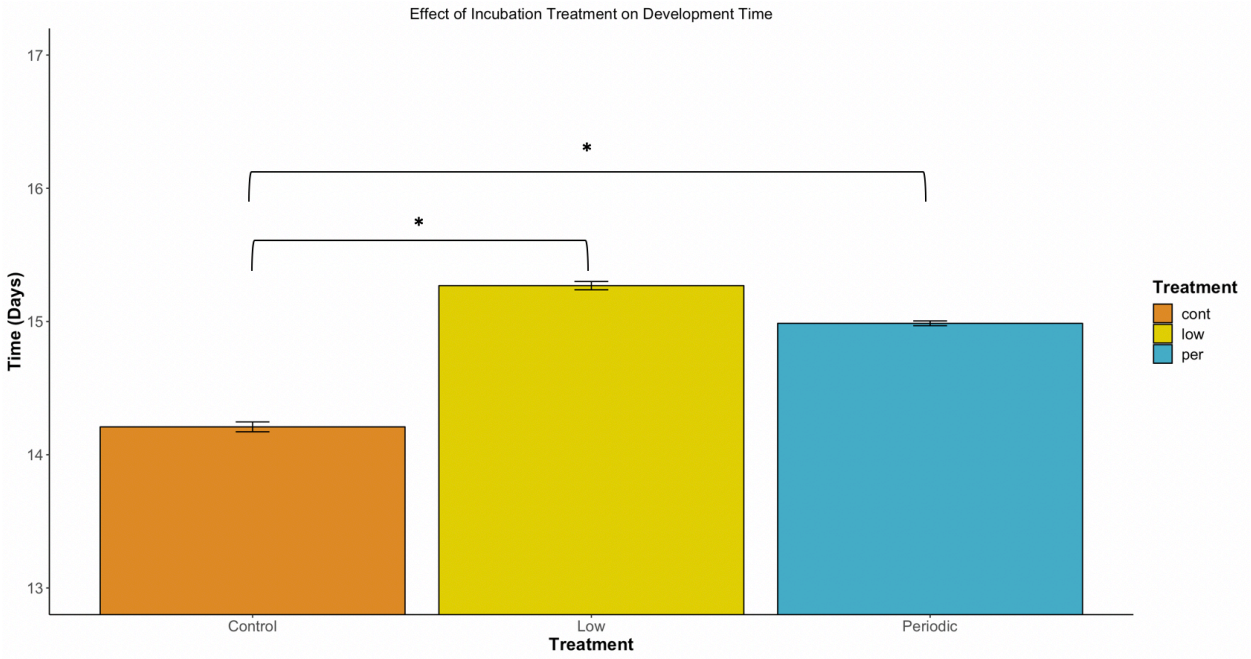

Figure 4. Effect of Incubation Treatment on Mean Development Time in Days. There were significant increases in incubation duration in both the Periodic and Low treatments.

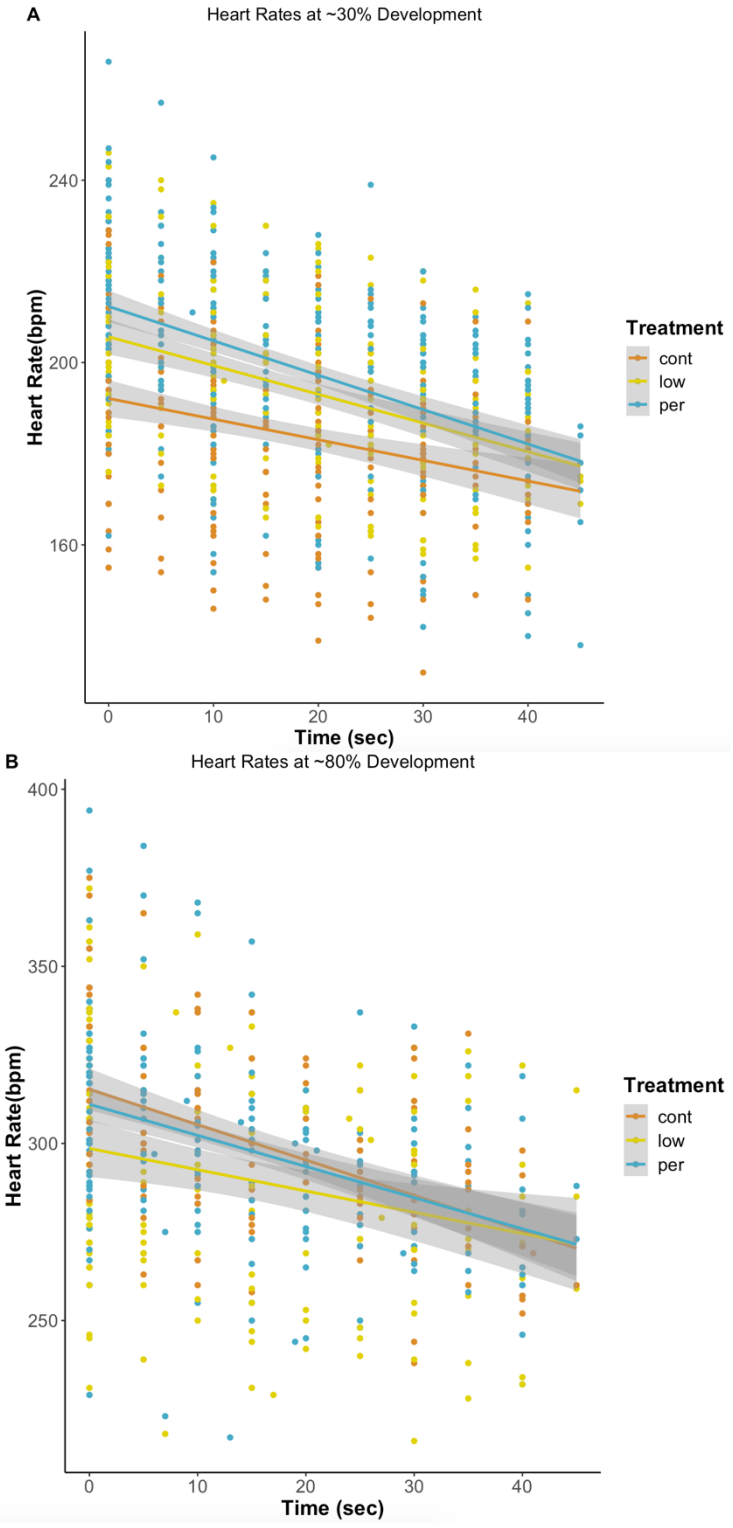

Figure 5. Effects of Incubation Treatment on Embryonic Heart Rates. **A**) Left centered embryonic heart rates at ~30% development **B)** Left centered embryonic heart rates at ~80% development. Shaded regions indicate standard error.

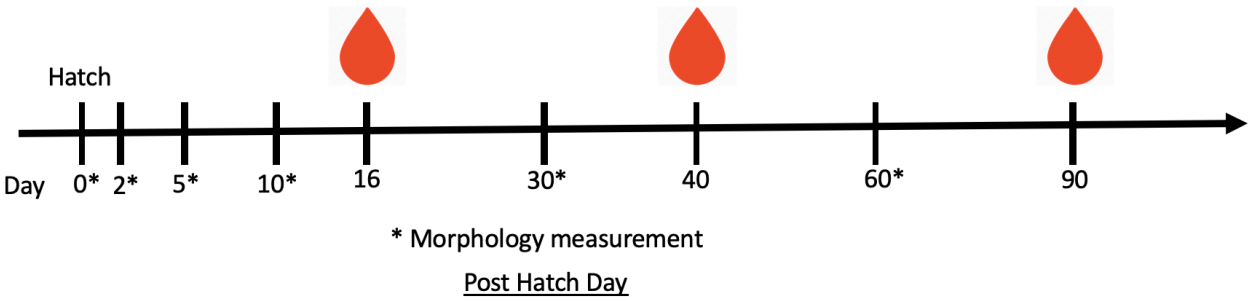

Figure 6. Post-hatch experimental timeline. Blood samples were taken on 16, 40, and 90 dph (indicated by red drops). Morphological measures of mass and/or tarsus were taken on days indicated by *.

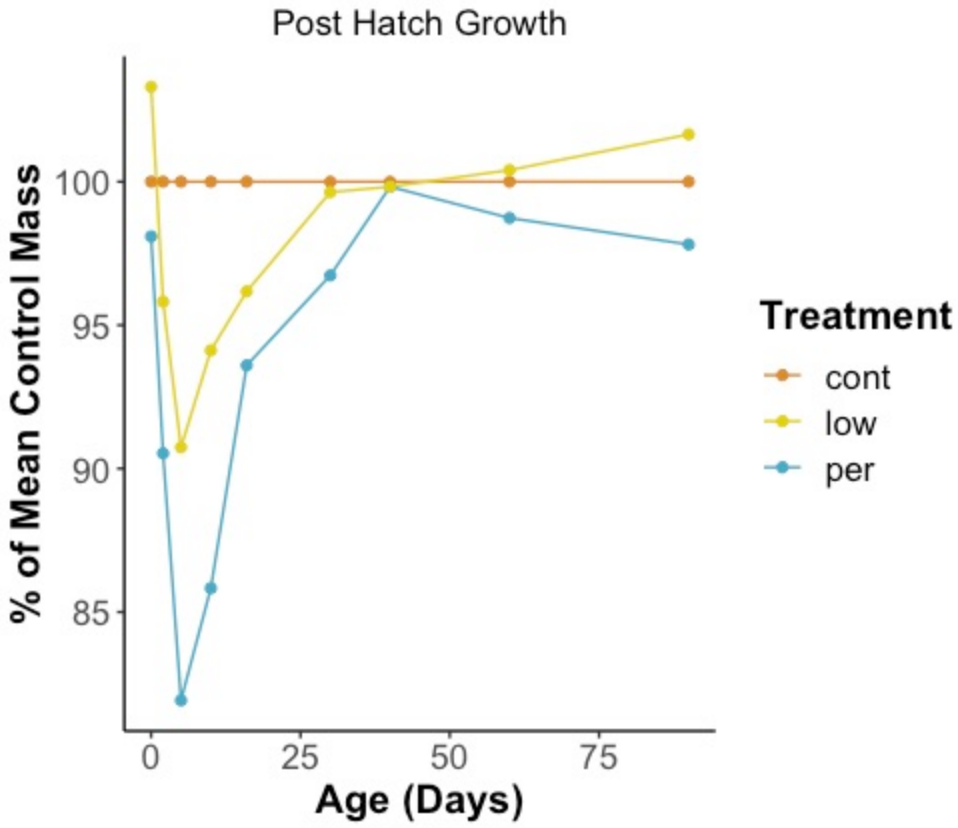

Figure 7: Percent change in zebra finch M_b in regard to treatment over the course of post-hatch development. Lines represent the percentage of the Low (yellow) and Periodic (blue) treatment compared to the Control group (orange).

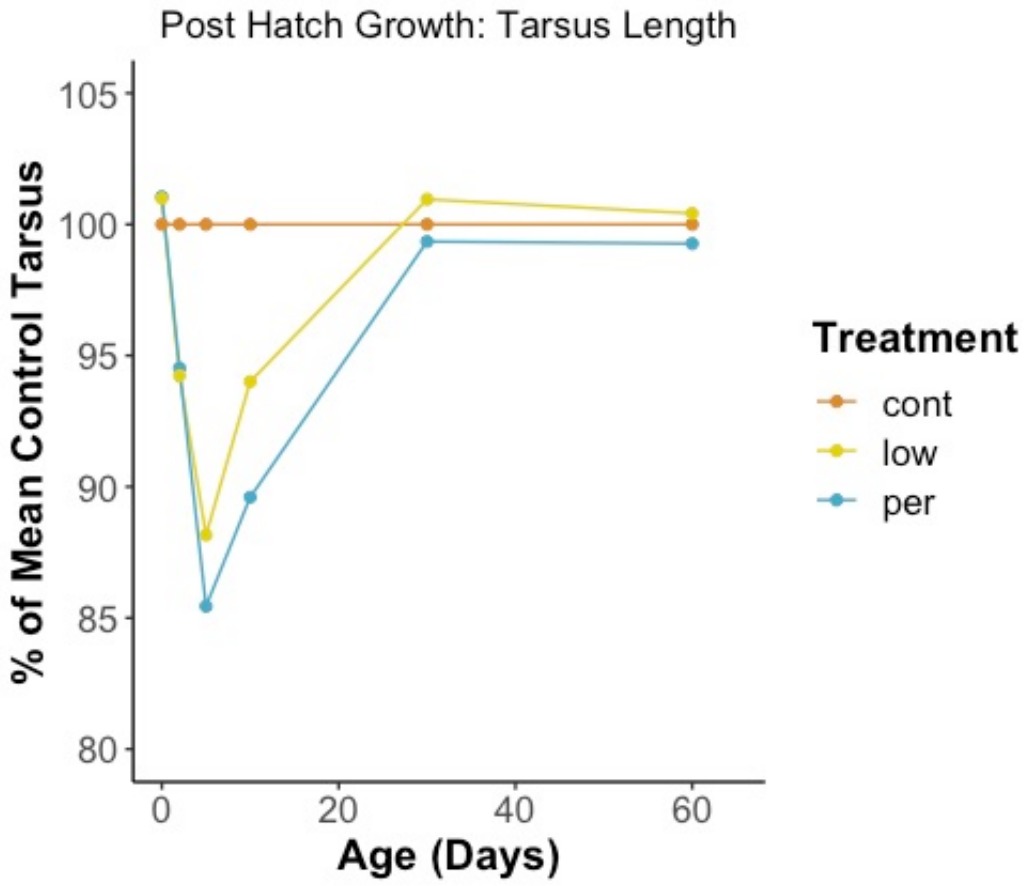

Figure 8. Percent change in zebra finch tarsus length in regard to treatment over the course of post-hatch development. Lines represent the percentage of the Low (yellow) and Periodic (blue) treatment compared to the Control group (orange).

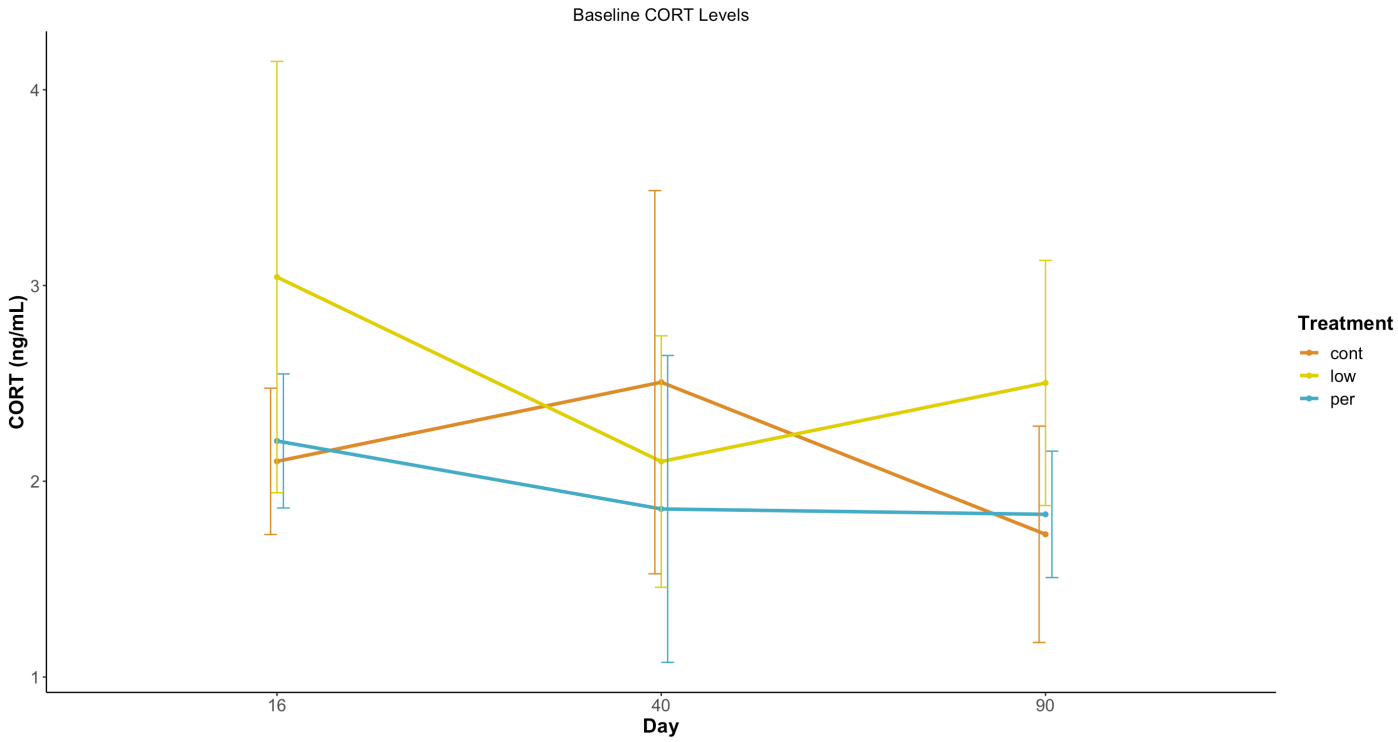

Figure 9. Baseline CORT levels of all treatments over time. There was no difference between treatments in baseline CORT levels at any time. Standard error bars are offset for clarity.

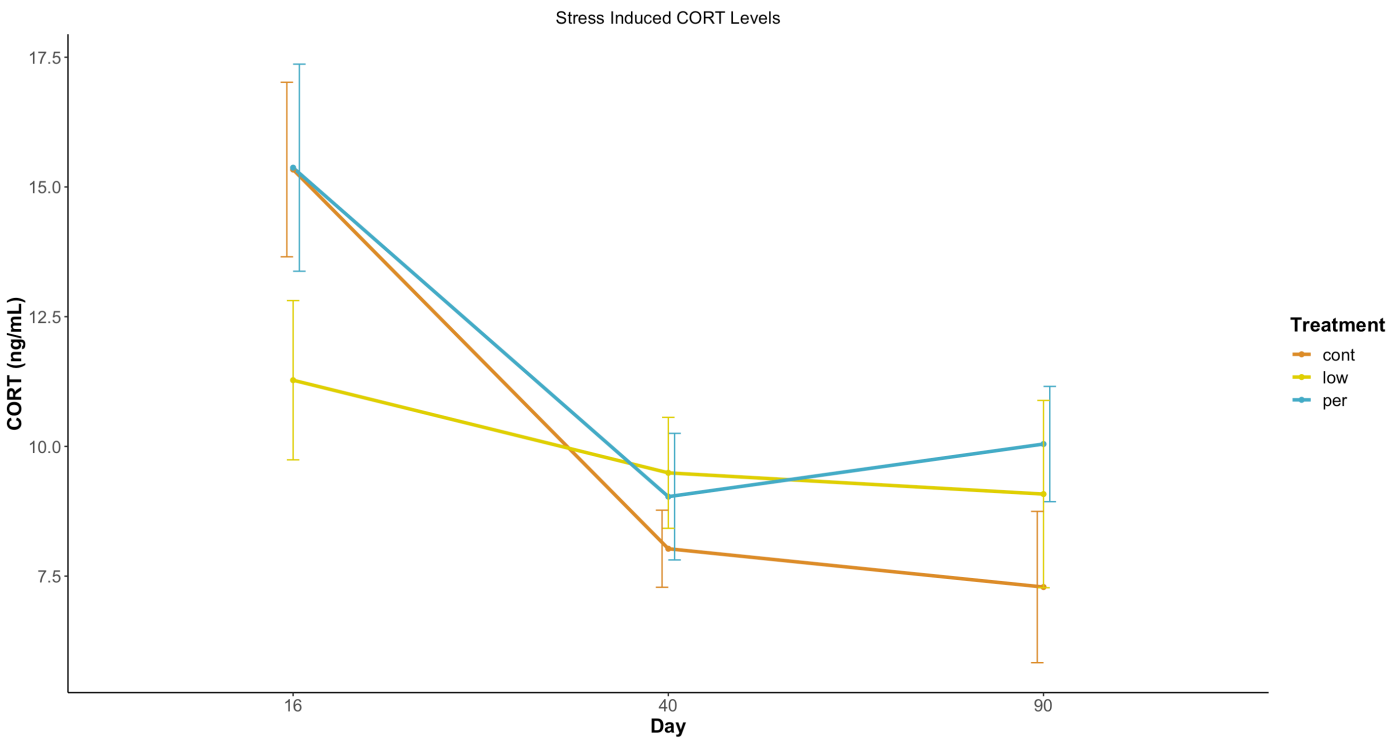

Figure 10. Stress induced CORT levels of all treatment over time. There was no difference in stress induced CORT levels at any time. Standard error bars are offset for clarity

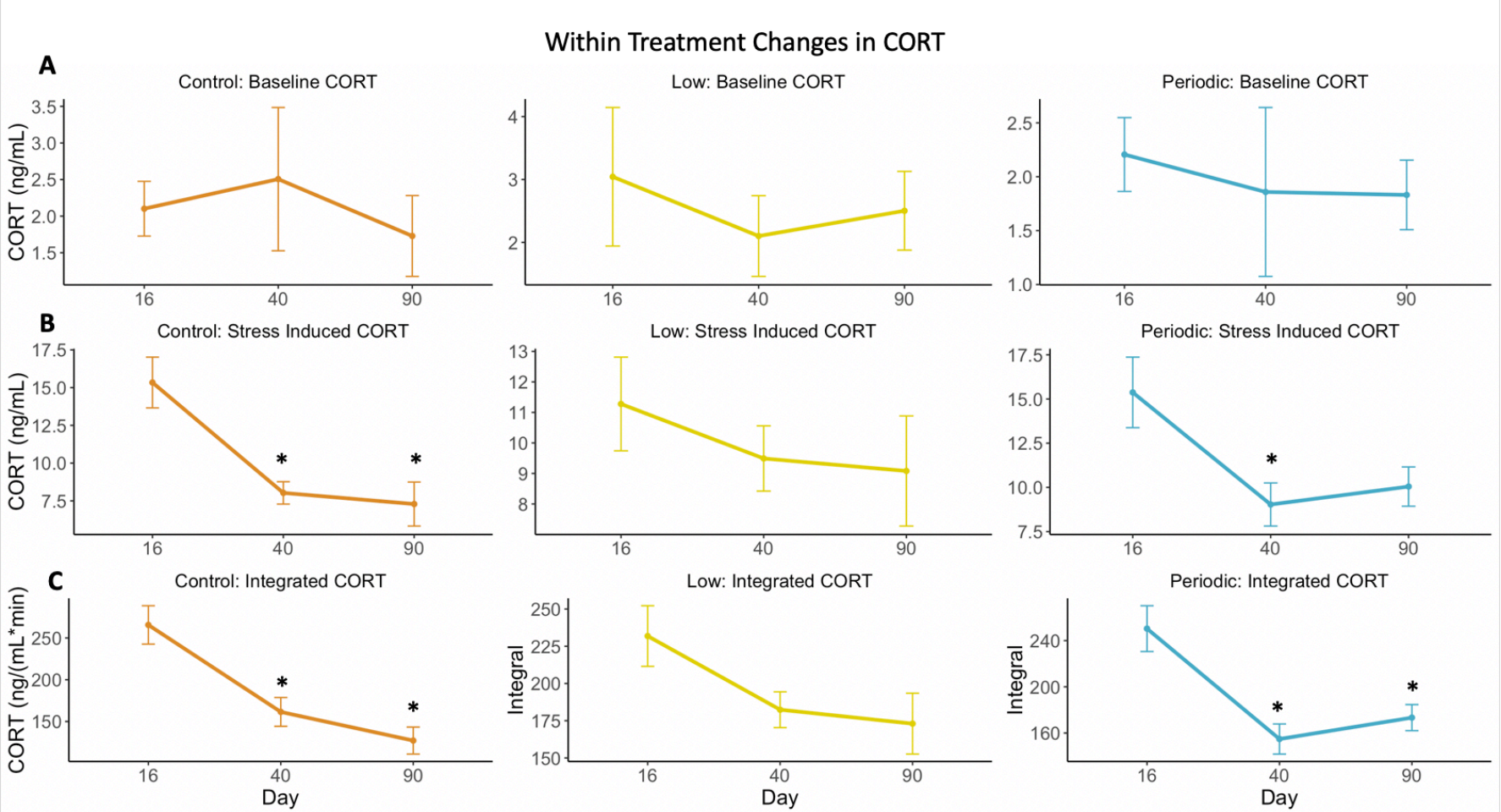

Figure 11. Within treatment changes in CORT for baseline (**A**), stress induced (**B**), and integrated response (**C**) levels over time. There were no changes in baseline CORT levels with age in any treatment. Stress induced levels significantly decreased at 40 dph in the Periodic group, and at 40 and 90 dph in the Control group. The integrated CORT response was significantly diminished at 40 and 90 dph in the Control and Periodic groups. * indicates differences from 16 dph within a treatment group.

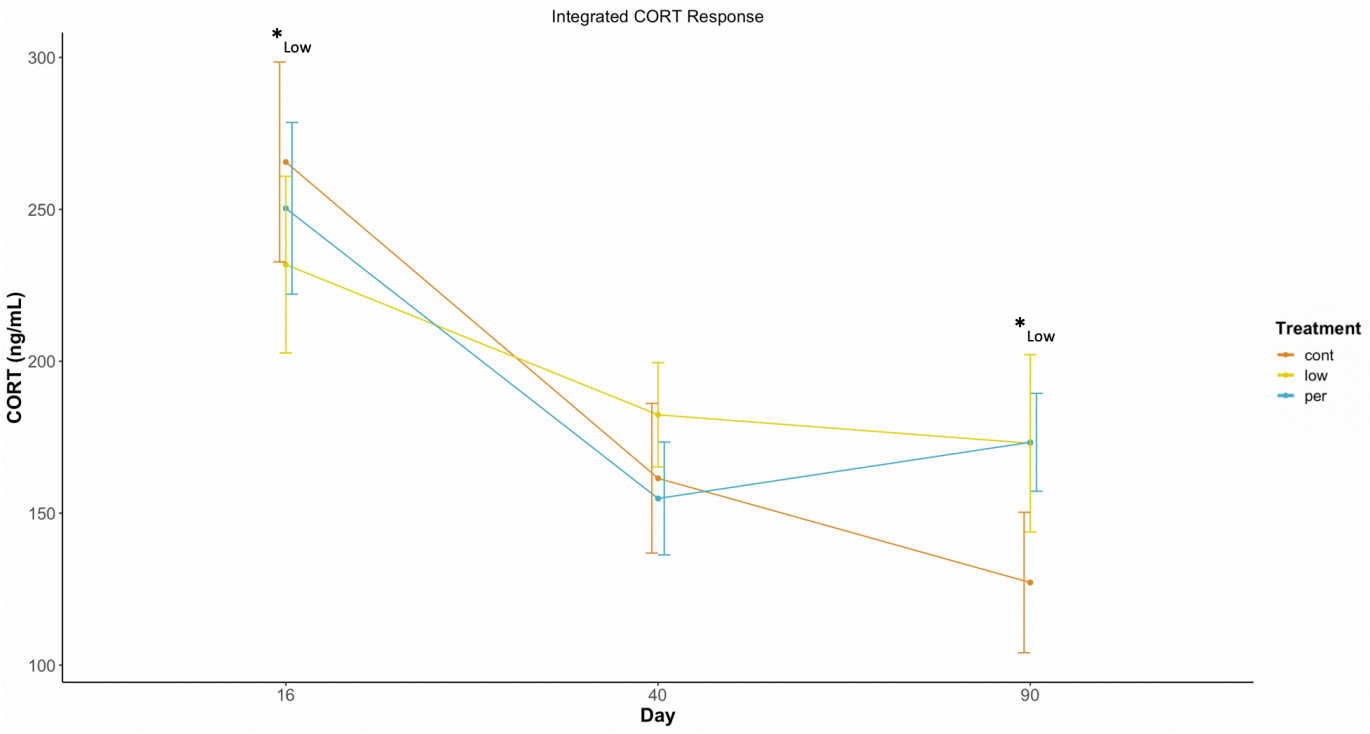

Figure 12. Changes in the integrated CORT response with age in regard to incubation treatment (effect of mass not included in graph). * represents significant difference from the Control group at that timepoint. Standard error bars are offset for clarity.

References

1. Angilletta M.J., R.S. Winters, and A.E. Dunham. 2000. Thermal effects on the energetics of lizard embryos: Implications for hatchling phenotypes. Ecology 81:2957–2968.
2. Ar A. and H. Tazawa. 1999. Analysis of heart rate in developing bird embryos: effects of developmental mode and mass. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 124:491–500.
3. Ardia D.R., J.H. Pérez, E.K. Chad, M.A. Voss, and E.D. Clotfelter. 2009. Temperature and life history: experimental heating leads female tree swallows to modulate egg temperature and incubation behaviour. Journal of Animal Ecology 78:4–13.
4. Ardia D.R., J.H. Pérez, and E.D. Clotfelter. 2010. Experimental cooling during incubation leads to reduced innate immunity and body condition in nestling tree swallows. Proceedings of the Royal Society B: Biological Sciences 277:1881–1888.
5. Beaman J.E., C.R. White, and F. Seebacher. 2016. Evolution of Plasticity: Mechanistic Link between Development and Reversible Acclimation. Trends in Ecology & Evolution 31:237–249.
6. Ben-Ezra N. and G. Burness. 2017. Constant and Cycling Incubation Temperatures Have Long-Term Effects on the Morphology and Metabolic Rate of Japanese Quail. Physiological and Biochemical Zoology 90:96–105.
7. Berntsen H.H. and C. Bech. 2016. Incubation temperature influences survival in a small passerine bird. Journal of Avian Biology 47:141–145.
8. Booth D.T. 2018. Incubation temperature induced phenotypic plasticity in oviparous reptiles: Where to next? Journal of Experimental Zoology Part A: Ecological and Integrative Physiology 329:343–350.

9. Bozinovic F., D.A. Bastías, F. Boher, S. Clavijo-Baquet, S.A. Estay, and M.J. Angilletta. 2011. The Mean and Variance of Environmental Temperature Interact to Determine Physiological Tolerance and Fitness. Physiological and Biochemical Zoology 84:543–552.
10. Burness G., J.R. Huard, E. Malcolm, and G.J. Tattersall. 2013. Post-hatch heat warms adult beaks: irreversible physiological plasticity in Japanese quail. Proceedings of the Royal Society B: Biological Sciences 280:20131436–20131436.
11. Careau V. and R.S. Wilson. 2017. Performance trade-offs and ageing in the 'world's greatest athletes.' Proceedings of the Royal Society B: Biological Sciences 284:20171048.
12. Carter A.W., W.A. Hopkins, I.T. Moore, and S.E. DuRant. 2014. Influence of incubation recess patterns on incubation period and hatchling traits in wood ducks *Aix* sponsa. Journal of Avian Biology 45:273–279.
13. Conway C.J. 2000. Effects of ambient temperature on avian incubation behavior. Behavioral Ecology 11:178–188.
14. Crino O.L. and C.W. Breuner. 2015. Developmental stress: evidence for positive phenotypic and fitness effects in birds. Journal of Ornithology 156:389–398.
15. Criscuolo F., P. Monaghan, L. Nasir, and N.B. Metcalfe. 2008. Early nutrition and phenotypic development: 'catch-up' growth leads to elevated metabolic rate in adulthood. Proceedings of the Royal Society B: Biological Sciences 275:1565–1570.
16. Dantzer B. and E.M. Swanson. 2012. Mediation of vertebrate life histories via insulin-like growth factor-1. Biological Reviews 87:414–429.

17. Dingemanse N.J. and N.A. Dochtermann. 2013. Quantifying individual variation in behaviour: mixed-effect modelling approaches. (M. van de Pol, ed.)Journal of Animal Ecology 82:39–54.
18. Du W.-G. and R. Shine. 2015. The behavioural and physiological strategies of bird and reptile embryos in response to unpredictable variation in nest temperature: Thermal response of reptile and bird embryos. Biological Reviews 90:19–30.
19. Du W.-G., M.-C. Tu, R.S. Radder, and R. Shine. 2013. Can Reptile Embryos Influence Their Own Rates of Heating and Cooling? (A. A. Romanovsky, ed.) PLoS ONE 8:e67095.
20. DuRant S.E., W.A. Hopkins, D.M. Hawley, and G.R. Hepp. 2012. Incubation temperature affects multiple measures of immunocompetence in young wood ducks (*Aix sponsa*). Biology Letters 8:108–111.
21. DuRant S.E., W.A. Hopkins, and G.R. Hepp. 2011. Embryonic Developmental Patterns and Energy Expenditure Are Affected by Incubation Temperature in Wood Ducks (*Aix sponsa*). Physiological and Biochemical Zoology 84:451–457.
22. DuRant S.E., W.A. Hopkins, G.R. Hepp, and J.R. Walters. 2013. Ecological, evolutionary, and conservation implications of incubation temperature-dependent phenotypes in birds: Incubation temperature and avian offspring phenotype. Biological Reviews 88:499–509.
23. Eiby Y.A. and D.T. Booth. 2009. The effects of incubation temperature on the morphology and composition of Australian Brush-turkey (*Alectura lathami*) chicks. Journal of Comparative Physiology B 179:875–882.

24. Gurley B., J.W. Finger, and H. Wada. 2018. Sex-Specific Effects of Incubation Temperature on Embryonic Development of Zebra Finch (*Taeniopygia guttata*) Embryos. Physiological and Biochemical Zoology 91:1036–1045.

25. Hall J.M. and D.A. Warner. 2018. Thermal spikes from the urban heat island increase mortality and alter physiology of lizard embryos. The Journal of Experimental Biology 221:jeb181552.

26. Hepp G.R., R.A. Kennamer, and M.H. Johnson. 2006. Maternal effects in Wood Ducks: incubation temperature influences incubation period and neonate phenotype. Functional Ecology 20:308–314.

27. Hoffman A.J., J.W. Finger, and H. Wada. 2018. Early stress priming and the effects on fitness-related traits following an adult stress exposure. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology 329:323–330.

28. Huey R.B., M.R. Kearney, A. Krockenberger, J.A.M. Holtum, M. Jess, and S.E. Williams. 2012. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philosophical Transactions of the Royal Society B: Biological Sciences 367:1665–1679.

29. Jimeno B., M. Briga, M. Hau, and S. Verhulst. 2018. Male but not female zebra finches with high plasma corticosterone have lower survival. (T. Williams, ed.)Functional Ecology 32:713–721.

30. Koláčková M., L. Prokůpková, T. Albrecht, and D. Hořák. 2015. Incubation Temperature Influences Trade-Off between Structural Size and Energy Reserves in Mallard Hatchlings. Physiological and Biochemical Zoology 88:1–10.

31. Lawson C.R., Y. Vindenes, L. Bailey, and M. van de Pol. 2015. Environmental variation and population responses to global change. (J. Drake, ed.)Ecology Letters 18:724–736.

32. Lepczyk C.A. and W.H. Karasov. 2000. Effect of ephemeral food restriction on growth in house sparrows. The Auk 117:164.
33. Liang L., B.-J. Sun, L. Ma, and W.-G. Du. 2015. Oxygen-dependent heat tolerance and developmental plasticity in turtle embryos. Journal of Comparative Physiology B 185:257–263.
34. Love O.P., P.O. McGowan, and M.J. Sheriff. 2013. Maternal adversity and ecological stressors in natural populations: the role of stress axis programming in individuals, with implications for populations and communities. (R. Boonstra, ed.)Functional Ecology 27:81–92.
35. Lynn S.E. and M.D. Kern. 2017. Ecologically Relevant Cooling Early in Life Alters Prefledging Adrenocortical Response in Free-Living Songbirds. Physiological and Biochemical Zoology 90:118–123.
36. Lynn S.E., M.D. Kern, and M.M. Phillips. 2013. Neonatal handling alters the development of the adrenocortical response to stress in a wild songbird (eastern bluebird, *Sialia sialis*). General and Comparative Endocrinology 186:157–163.
37. correlations? General and Comparative Endocrinology 190:188–193.
38. Monaghan P. 2008. Early growth conditions, phenotypic development and environmental change. Philosophical Transactions of the Royal Society B: Biological Sciences 363:1635–1645.
39. Noble D.W.A., V. Stenhouse, and L.E. Schwanz. 2018. Developmental temperatures and phenotypic plasticity in reptiles: a systematic review and meta-analysis: Incubation temperature and plasticity. Biological Reviews 93:72–97.
40. Nord A. and J.-Å. Nilsson. 2011. Incubation Temperature Affects Growth and Energy Metabolism in Blue Tit Nestlings. The American Naturalist 178:639–651.

41. Olson C.R., C.M. Vleck, and D.C. Adams. 2008. Decoupling morphological development from growth in periodically cooled zebra finch embryos. Journal of Morphology 269:875–883.
42. Olson C.R., C.M. Vleck, and D. Vleck. 2006. Periodic Cooling of Bird Eggs Reduces Embryonic Growth Efficiency. Physiological and Biochemical Zoology 79:927–936.
43. Plewis, I. 1989 Comment on "centering" predictors in multilevel analysis. Multilevel Modelling Newletter 1: 6-11.
44. Reyna K.S. and W.W. Burggren. 2017. Altered embryonic development in northern bobwhite quail (*Colinus virginianus*) induced by pre-incubation oscillatory thermal stresses mimicking global warming predictions. (A. Yildirim, ed.)PLOS ONE 12:e0184670.
45. Romero L.M. and M. Wikelski. 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. Proceedings of the Royal Society B: Biological Sciences 277:3157–3162.
46. Romero L.M., M.J. Dickens, and N.E. Cyr. 2009. The reactive scope model — A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375–389.
47. Sapolsky R.M., L.M. Romero, and A.U. Munck. 2000. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions 21:35.
48. Schmidt K.L., E.A. MacDougall-Shackleton, and S.A. MacDougall-Shackleton. 2012. Developmental stress has sex-specific effects on nestling growth and adult metabolic rates but no effect on adult body size or body composition in song sparrows. Journal of Experimental Biology 215:3207–3217.

49. Schmidt K.L., E.A. MacDougall-Shackleton, K.K. Soma, and S.A. MacDougall-Shackleton. 2014. Developmental programming of the HPA and HPG axes by early-life stress in male and female song sparrows. General and Comparative Endocrinology 196:72–80.

50. Schneider C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675.

51. Sheldon E.L., L.S.C. McCowan, C.S. McDiarmid, and S.C. Griffith. 2018. Measuring the embryonic heart rate of wild birds: An opportunity to take the pulse on early development. The Auk 135:71–82.

52. Tiatragul S., A. Kurniawan, J.J. Kolbe, and D.A. Warner. 2017. Embryos of non-native anoles are robust to urban thermal environments. Journal of Thermal Biology 65:119–124.

53. Wada H., S.E. DuRant, and F.M. Anne McNabb. 2018. Altricial and Precocial Development in Birds. Pp. 50–56 in Encyclopedia of Reproduction. Elsevier.

54. Wada H., B. Kriengwatana, N. Allen, K.L. Schmidt, K.K. Soma, and S.A. MacDougall-Shackleton. 2015. Transient and permanent effects of suboptimal incubation temperatures on growth, metabolic rate, immune function and adrenocortical responses in zebra finches. Journal of Experimental Biology 218:2847–2855.

55. Wada H., K.G. Salvante, E. Wagner, T.D. Williams, and C.W. Breuner. 2009. Ontogeny and Individual Variation in the Adrenocortical Response of Zebra Finch (*Taeniopygia guttata*) Nestlings. Physiological and Biochemical Zoology 82:325–331.

56. Warner D.A. and R. Shine. 2011. Interactions among thermal parameters determine offspring sex under temperature-dependent sex determination. Proceedings of the Royal Society B: Biological Sciences 278:256–265.

57. Westneat D.F., M.I. Hatch, D.P. Wetzel, and A.L. Ensminger. 2011. Individual Variation in Parental Care Reaction Norms: Integration of Personality and Plasticity. The American Naturalist 178:652–667.
58. While G.M., D.W.A. Noble, T. Uller, D.A. Warner, J.L. Riley, W.-G. Du, and L.E. Schwanz. 2018. Patterns of developmental plasticity in response to incubation temperature in reptiles. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology 329:162–176.
59. Wyneken J. and A. Lolavar. 2015. Loggerhead sea turtle environmental sex determination: Implications of moisture and temperature for climate change based predictions for species survival: environmental sex determination in in loggerhead turtles. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 324:295–314.
60. Zann R. and M. Rossetto. 1991. Zebra Finch Incubation: Brood patch, Egg Temperature and Thermal Properties of the Nest. Emu - Austral Ornithology 91:107–120.