INCUBATION TEMPERATURE IMPACTS GROWTH, PHYSIOLOGY AND SURVIVAL IN NESTLINGS OF AN OPEN-CUP NESTING PASSERINE

BY

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THESIS

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ABSTRACT

Early-life conditions can have important implications for somatic and physiological development, and later life survival. For oviparous species, embryonic development occurs wholly within the egg. During this stage, there is good evidence that egg resources such as lipid and protein content and maternal hormones play a critical role in development. Much less is known, however, about the role of incubation temperature in shaping the phenotype. Among species in which one or both parents incubate, there is the possibility that changes in incubation behavior on the part of the parent(s) can impact the developing offspring. The vast majority of birds require bird-egg contact during incubation to maintain an appropriate environment for embryonic development. Therefore, understanding the ways in which incubation temperature influences variation in nestling growth and physiological development as well as survival may be of critical importance to bird conservation. I examined how variation in incubation temperature influences key aspects of nestling development in American robins (*Turdus migratorius*). Specifically, I sought to address whether incubation temperature influences (1) hatching success, (2) nestling growth rate and three key morphological characteristics, (3) development of the Hypothalamic-Pituitary-Adrenal (HPA) axis and aspects of innate immune defenses in nestling robins, as well as associations between HPA axis activity and immune activity, and (4) nestling survival. From March to August of 2015 and 2016, I worked in a mixed coniferous tree farm in central Illinois, USA, and tracked growth, development of the HPA axis (responsible for stress hormone production, corticosterone) and innate immunity, and survival of nestling robins that were incubated as eggs in one of three temperature categories: 36.1 °C, 37.8 °C, and Naturally incubated. Hatched nestlings from the experimental categories were fostered to same-age nests and monitored throughout the nestling period to determine fate. On days 7 and 10 post-hatch, I

measured mass and tarsus length and collected blood samples from experimentally incubated nestlings and one foster nest-mate (Naturally incubated). I collected data from 339 nests and 85 artificially incubated nestlings.

I found that nestlings from Low and Optimal incubation categories had lower mass and shorter tarsi, significant reductions in survival, and increased corticosterone concentration. I found no effect of incubation category on immune function, and no significant relationships between corticosterone concentration and innate immune function in nestling robins. My results suggest that incubation temperature can influence subsequent nestling growth, physiology, and survival. This research suggests that factors influencing incubation temperature are important for the fitness of nestlings. Thus, environmental disturbances that disrupt incubation may have population-level impacts. Collectively, my research adds to the growing body of literature suggesting the importance of sublethal stressors (e.g. reduced incubation temperature) on fitness and population dynamics and highlights the importance of these stressors in leading to such effects on nestling survival and development.

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CHAPTER 1: INCUBATION TEMPERATURE IMPACTS NESTLING GROWTH AND SURVIVAL IN AN OPEN-CUP NESTING PASSERINE

Abstract

Early developmental conditions have implications for later life fitness. For birds, the effects of subtle temperature changes during incubation on nestling development remain poorly understood. I investigated how incubation temperature affected morphological characteristics as well as nestling survival in American Robins (*Turdus migratorius*) in a mixed coniferous tree farm in Central Illinois, USA. More specifically, I sought to understand how stressors experienced by the embryo during the incubation period could carry over to the nestling period and alter an individual's phenotype and/or survival. Eggs were collected in the field and incubated in one of two temperature treatments: 36.1 °C and 37.8 °C. Lab-incubated nestlings were transported to the field and fostered to same-age nests following hatching. Nestlings were monitored every two days to determine mortality, and on days 7 and 10 post-hatch, I measured mass, tarsus and wing length of experimentally incubated nestlings and randomly selected foster nest-mate from naturally incubated (non-experimental) nests. Nestlings experiencing more stressful conditions (i.e. lower incubation temperatures) had lower mass and shorter tarsi and significant reductions in survival. My results suggest that nest microclimate (specifically temperature) influences nestling phenotype and survival. These findings indicate that incubation temperature, and subsequently factors influencing incubation temperature, are vitally important to the long-term fitness and survival of robins.

Introduction

Environmental conditions during development can impact both short- and long-term phenotypic expression (Weaver 2009). Variation in behavioral, physiological, and morphological traits have putative fitness-related consequences (West-Eberhard 2005). Consequently, disturbance during early-life periods, such as embryonic development, can have important fitness-related ramifications for offspring (Cain et al., 1967; Heintz et al. 1999; Booth 2006).

Temperature is one of the most important abiotic factors influencing animal behavior, physiology, and survival (Brett 1969). Thermal regime during development has been shown to influence offspring phenotype including, but not limited to, survival, hatchling size, post-hatch growth, locomotor performance, morphology, and sex ratios (Bull 1980, Rana 1990, Van Damme et al. 1992, Andrews et al. 2000). Oviparous species, for which development is generally entirely outside of the mother, are particularly sensitive to external factors such as temperature. For successful development, embryos often require a narrow range of temperatures (Webb 1987). In birds, incubation temperatures are mediated through behaviors and actions carried out by adults, including nest site location, construction of the nest, and incubation behavior (Deeming 2002). These behaviors can be broadly classified as parental effects, defined as the effects parents have on the expression of offspring phenotype that are unrelated to the offspring's own genotype (Uller 2008). Parental effects can be broken down into paternal and maternal effects, in which the former represents non-genetic influence of the father on the offspring's phenotype, and the latter is the mother's influence.

For many bird species, egg incubation is performed solely by the female, and thus represents a form of maternal effect. The incubation period plays a crucial role in adult bird reproduction, with most species exhibiting contact incubation to maintain a suitable environment

for embryonic growth and development (Deeming 2002). During this period, adult birds must balance current reproductive expenditures (e.g. demands of the developing embryo) against future reproductive potential (e.g. costs of self-maintenance) (Stearns 1992; Reid et al. 2002). Despite the variety of climates in which eggs are laid, there is a generally restricted thermal range in which avian embryos develop, which is typically between 38 and 39 ˚C (Carey 1980). Exposure to temperatures outside of this optimal range for extended periods can be deleterious to developing embryos (Lundy 1969). Decreases in nest attentiveness and reductions in incubation temperature are known to impact nestling development and survival in a number of ways, including: 1) extending the development period, thereby prolonging the time in which a nest is susceptible to predation (Martin 2002), 2) reducing hatching success and survival of young (DuRant et al. 2010), and 3) retarding morphological development of nestlings (Webb 1987; Nord and Nilsson 2011). There has been substantial work on the effects of incubation temperature in the poultry industry (e.g. Michels et al. 1974, Hulet et al. 2007, Leksrisompong et al. 2007, Nangsuay et al. 2016), however, past research on the effects of incubation temperature in wild birds appears to be limited to cavity nesting species, and/or species with precocial offspring (Hepp et al. 2006; DuRant et al. 2010; Nord and Nilsson 2011). These species are amenable for studies examining the effects of changes in incubation temperature on growth and development, but the majority of bird species are open-cup nesting species with altricial young. To the best of my knowledge, no work has been done on the effects of changes in incubation temperature on development and survival of open-cup nesting species with altricial young. These species differ from cavity-nesters and precocial species in a couple of important respects. Many cavity nesting species take frequent foraging trips and have relatively short on- and off-bouts during the incubation period compared to open-cup nesting species (Conway and Martin 2000).

These short off-bout periods are thought to facilitate more stable incubation temperatures that are less affected by environmental fluctuations. Open-cup nesting birds are exposed to higher rates of predation (Lack 1954; Nice 1957; Ricklefs 1969) and greater variability in temperature throughout the nesting period (Heenan 2013), which may profoundly impact grown, development, and survival.

I examined the effects of incubation temperature on the length of the incubation period, hatching success, development, and survival of American robin (*Turdus migratorius*; hereafter robin(s)) nestlings incubated under experimental and natural conditions. I hypothesized that incubation temperature would impact development and probability of survival in nestling robins. Specifically, I predicted that (1) incubation duration would vary inversely with incubation temperature (2) nestlings incubated at experimentally controlled, suboptimal temperatures would be smaller and exhibit reduced survival compared to naturally incubated individuals and (3) individuals incubated optimal conditions would not differ in survival or development from those incubated naturally.

Methods

Study Site and Species

My study was conducted on an 8 ha mixed coniferous tree farm located near Urbana, Illinois, USA (40°02′N, 88°10′W) during the 2015 and 2016 breeding seasons. The site consisted mainly of Eastern white pine (*Pinus strobus*), Wite fir (*Abies concolor*), Blsam fir (*Abies balsamea*), Fraser fir (*Abies fraseri*), Douglas fir (*Pseudotsuga menziesii*), and Scots pine (*Pinus sylvestris*) ranging in age from newly planted saplings to 16 years old. Trees were planted in discrete rows which allowed for rapid and thorough nest searching. The most common species observed breeding on the site were northern cardinals (*Cardinalis cardinalis*), house finches

(*Haemorhous mexicanus*), common grackles (*Quiscalus quiscula*), and American robins. I chose robins as my model species as they are found in high densities, are multi-brooded, and exhibit uniparental incubation. I located nests by systematically searching every tree on the site every other day. Nests found during the building and laying periods were checked every day to ensure an accurate clutch completion date. All other nest-checks were conducted every other day in an attempt to minimize disturbance to the birds, while still obtaining high-resolution information on fate. Incubating birds typically returned to the nest within 2-3 minutes following nest-check.

Incubation Patterns and Analysis

To determine the temperature and incubation patterns of robin nests, I placed a small metallic temperature logger amongst the eggs (Thermocron iButtons DS1921G, Maxim, San Jose, California) after clutch completion. iButtons were affixed to the nest using a combination of Velcro, a shirt button, and pliable wiring so as to be comparable to the eggs in both height and position within the nest and ensure direct contact with the brood patch. This setup ensured that iButton placement within the nest was consistent throughout incubation, and prevented females from removing or burying them in the nest. iButtons were also colored blue using a permanent marker that most closely resembled the color of robin eggs. Temperature was recorded at 2-min intervals, and the data were downloaded and iButtons reprogrammed about every 2.5 days. I used Rhythm 1.1 (Cooper and Mills 2005) to automate the selection of on- and off-bouts, and Raven Pro 1.4 (Bioacoustics Research Program, Cornell University, Ithaca, New York) to assess and modify selections when necessary. Rhythm was used to transform iButton data into useable sound files and to automate selections of on- and off-bouts. Raven was used to visually assess accuracy of automated selection and to classify selections (i.e. on- vs off-bouts). These processing steps allowed me to estimate both temperature and duration of on- and off-bouts.

Egg Collection, Incubation, and Cross Fostering

Nests that were located during the building or laying phase and that contained ≥3 eggs were deemed suitable for egg collection and were considered focal nests. Prior to the onset of incubation, I randomly selected, removed, and marked one egg from each focal nest. Eggs were placed in cotton lined Falcon 50 mL Conical Centrifuge Tubes to provide a safe transportation environment for the eggs. Collected eggs were randomly placed into one of two temperature categories: 36.1 °C (presumed suboptimal incubation temperature; Lundy 1969; hereafter "Low treatment") and 37.8 °C (presumed optimal incubation temperature; Kuehler and Good 1990; hereafter "Optimal treatment") both set to 60-65% relative humidity (Kuehler and Good 1990). Early on in the experiment my "Low" incubation temperature was 35 °C, but during the initial round of egg collections, it became evident that 35 °C was too low for robin eggs (0% hatching success, $n = 11$). Using both the ovascope and by dissecting eggs, I determined that hatching failure was caused by embryonic death late in development, possibly caused by nutritional stress or delayed development of the hatching muscle (Olson et al. 2006, Olson et al. 2008). At this point, I increased the temperature to 36.1 °C. Incubators (Turn X7, Lyons USA, Chula Vista, Ca.) were housed at the University of Illinois, where they were closely monitored to ensure proper incubation temperature and humidity throughout the experiment. I monitored progression of embryonic development during incubation using an ovascope (Brinsea OvaScope Egg Viewer, Titusville, FL.). Two days prior to hatching, I transferred the eggs to a non-turning incubator to ensure a safe hatching environment. Once hatched, nestlings were massed (only in 2016), marked using a non-toxic permanent marker, transferred to the field in a small cooler warmed with rechargeable handwarmers, and placed in a non-natal nest with three or four same age

nestlings. By cross-fostering nestlings, I hoped to isolate the effects of incubation temperature from any genetic and maternal effects.

Nestling Sampling

Experimental nestlings were remarked every other day with a non-toxic permanent marker during the nest check until banding to ensure accurate identification of the experimental nestling throughout the experiment. On day 7 post-hatch, I banded each experimentally incubated nestling (hereafter "focal nestling") and a randomly selected naturally incubated nestling of the foster nest (hereafter "foster nest-mate"). On days 7 and 10 post-hatch, I measured mass, and wing and tarsus length for each focal nestling and its associated foster nest-mate. I also collected a small sample of blood (< 0.05% blood volume) prior to and following the administration of a standardized 30 min. stress protocol (Breuner et al. 1999) as part of another study, and immediately returned the nestlings to the nest. Due to time constraints, only the focal nestling and foster nest-mate were sampled and banded.

Statistical Methods

I estimated baseline-hatching success for robin eggs using only those nests that were not manipulated (i.e. no egg removed or added, and no iButton) within this study system. I used generalized linear mixed models (SAS PROC GLIMMIX; binomial distribution, logit link function) to estimate the probability of a nestling surviving to day 7 or 10 post-hatch for a given treatment including nest and nestling identity as random effects in all models when appropriate. I examined morphological differences among temperature categories (Low, Optimal, Natural) using a general linear mixed model (Low, Optimal, and Natural; SAS PROC MIXED), as well as differences between a given focal nestling and its foster nest-mate using a paired t-test (SAS PROC TTEST) to control for parental effects (e.g., provisioning and nestling incubation). I

estimated the growth rate of each morphological character for each nestling by calculating the difference in measurements between day 7 and 10 post-hatch. I then compared growth rates among temperature categories (Low, Optimal, Natural) using a general linear mixed model. I estimated clutch survival (total and partial clutch loss) using generalized linear mixed models (binomial distribution, logit link function) including a random effect of nest identity. All statistical tests were performed using SAS 9.4.

Results

Incubation Period Length and Hatching Success

Across both sampling years, I located and monitored a total of 339 nests. Hatching success for non-manipulated nests (n = 87) was 85.2% (226 eggs hatched out of a total of 265 eggs laid). Following the onset of incubation, nest temperatures varied widely from a low of -2.4˚C to a high of 45 ˚C during extreme periods, but tended towards temperatures within a range of more optimal development (37˚C - 38˚C; Table 1; Fig. 1). Hatching success for eggs from the artificial incubators was comparable, although slightly higher (Low treatment (36.1˚C): 89.2%; Optimal treatment (37.8˚C): 90.2%), but differences in hatching success among treatments were not statistically significant (p = 0.452, Fig. 2).

Artificial incubation significantly decreased the length of the incubation period compared to Naturals ($F_{2,124}$ = 31.5; p < 0.0001; Fig. 3). Length of the incubation period was more similar between Low treatment and naturally incubated eggs (differing by 0.45 days, p = 0.038) than between those incubated in the Optimal treatment and naturally incubated eggs (differing by 1.73 days, p < 0.0001) although both differed significant from the naturally incubated nestlings. Optimal treatment incubated eggs experienced shorter incubation periods compared to Low treatment incubated eggs (differing by 1.28 days; p = 0.038).

Nestling Survival and Morphology

Incubation category had a significant effect on survival to day 7 post-hatch, but not on day 10 post-hatch (day 7: $F_{2,623} = 4.83$; $p = 0.008$; day 10: $F_{2,623} = 1.64$; $p = 0.195$). Naturally, incubated nestlings had higher probability of survival to days 7 and 10 post-hatch compared to both Optimal and Low treatment nestlings (Fig. 2). Optimal-treatment nestlings exhibited intermediate levels of survival to days 7 and 10 post-hatch which were not significantly lower than naturally incubated nestlings, and marginally significantly higher than Low treatment nestlings (Natural vs Optimal: $p = 0.275$; Optimal vs Low: $p = 0.093$; Fig. 2). Nests that received an artificially incubated nestling were significantly more likely to experience total clutch loss than non-manipulated nests ($F_{1,457} = 3.84$; $p = 0.050$). Rates of total clutch loss did not differ between the two experimental treatments (36.1 ˚C and 37.8 ˚C; $F_{1,456} = 0.43$; $p = 0.664$). There was, however, a trend for partial clutch loss to vary by experimental treatment, in which nests that contained a Low-treatment nestling were more likely to experience partial clutch loss than nests with an Optimal-treatment nestling (75% loss from 36.1 ˚C and 15% loss from 37.8 ˚C; $F_{1,12} = 4.56$ $p = 0.052$).

At days 7 and day 10 post-hatch, mean mass and tarsus length were smallest for nestlings incubated at 36.1 °C, and largest for nestlings reared under natural conditions (Fig. 4). Focal chicks from both experimental treatments were significantly smaller in mass, wing, tarsus length than their foster nest-mates on day 7 post-hatch (Table 2) but that difference only persisted for the focal chicks from the Optimal treatment to day 10 post-hatch in mass and tarsus length. On day 10 post-hatch, focal nestlings from the Low treatment no longer differed significantly in mass or tarsus length from their foster nest-mate.

Nestlings that hatched from artificially incubated eggs (either experimental treatment) tended to exhibit larger growth rates than their foster counterparts, but rates were not significantly different for any of the morphometric measurements (Table 4; Fig. 5).

Discussion

For oviparous species, conditions experienced while in the egg have profound, and potentially long-lasting effects on the organism (Spotila et al. 1994; Shine et al. 1997). For many birds, incubation temperature has been presumed to play an important role in development, although there has been little work conducted on wild birds (but see DuRant et al. 2010; Nord and Nilsson 2011; DuRant et al. 2013). Here I demonstrate that differences in incubation temperature within the natural range of variation can have large-scale effects on growth and survival in a wild bird. These effects manifested in lengthening of the incubation period, lower survival, and smaller average body size at 'Low' temperatures (i.e. 36.1 °C) compared to nestlings from 'Optimal' (i.e. 37.8 °C) and Natural conditions. Furthermore, although within the physiological range for incubation and having been used in previous studies (Nord and Nilsson 2011; DuRant et al. 2013) my initial 'low' temperature category (i.e. 35 °C) resulted in embryonic death. Using both the ovascope and by dissecting eggs, I determined that hatching failure was caused by embryonic death late in development, possibly caused by nutritional stress or delayed development of the hatching muscle (Olson et al. 2006, Olson et al. 2008). Additionally, data from temperature loggers suggest that eggs in natural nests experience temperatures greater than 38 °C and do experience profound variation in temperature throughout the incubation period. Therefore, it may be that 'optimal' incubation temperature depends on some combination of a proper 'optimal' incubation temperature in conjunction with normal fluctuations in temperature produced by off-bouts that create the ideal environment for

embryonic development. Further complicating this already complex issue, there is a fine balance between an incubation period that is too long and one that is too short as evidenced by the embryos that never hatched from my first 'low' incubation temperature treatment (35 °C). My observations help reinforce other findings supporting links between incubation temperature and subsequent nestling condition (e.g., Reid et al. 2002; DuRant et al. 2010; Nord and Nilsson 2011).

In concert with previous research (DuRant et al. 2010; Nord and Nilsson 2011), I found that the incubation period was shortest for those eggs artificially incubated at the higher temperature. Shorter incubation periods could potentially be advantageous to both the adult and offspring by reducing the predation risk associated with additional days in the nest (Martin 2002). Increased attentiveness at the nest can manifest as higher average incubation temperatures (White and Kinney 1974), but can also lead to greater energetic and predation risk costs for the parent (Williams 1996; Ghalambor and Martin 2002). Breeding birds must balance the costs associated with current reproduction against the perceived value of current and future reproductive ventures, resulting in a tradeoff between investing in the current clutch and investing in future reproduction (i.e. self-maintenance) (Stearns 1992). For example, recent work suggests that in an environment where adult mortality is high it is in the best interest of the adult to maximize their current reproductive output through optimal incubation temperatures, specifically through parental time and effort allocated to warming the clutch after an off-bout (Martin et al. 2015).

While more rapid rates of development may be beneficial, there is likely some optimal rate of development, above which the offspring may suffer deleterious effects. Nestlings from both experimental treatments were smaller than nestlings from naturally incubated nests. I

predicted that nestlings incubated at 36.1 °C to be smaller than those incubated at higher temperatures, and expected similarity between nestlings incubated at 37.8 °C and under natural conditions given that ~38 °C is presumed to be optimal (Kuehler and Good 1990). The smaller experimentally incubated nestlings may have been caused by several factors. Experimentally incubated eggs were exposed to relatively constant temperatures whereas naturally incubated eggs experience temperature fluctuations throughout the incubation period; these fluctuations may play an important role in proper embryonic development, and can potentially impact nestling traits important for future development, survival, and reproduction (DuRant et al. 2013). Indeed, previous research suggests that slower development time caused by intrinsic mechanisms (e.g. rate of yolk consumption) can yield long-term benefits for offspring quality (Metcalfe and Monaghan 2003). Therefore, by maintaining eggs in a state of high and constant incubation temperature (particularly in the Optimal treatment and to a lesser extent in the Low treatment), I potentially reduced offspring quality by forcing more rapid rates of embryonic development. Additionally, data from temperature loggers suggest that eggs in natural nests experience temperatures greater than 38 °C and do experience profound variation in temperature throughout the incubation period. Based on my data and the embryos that never hatched from my initial low-temperature treatment (35˚C), there is a fine balance between an incubation period that is too long and one that is too short. It is worth noting that domestic chicken eggs that are typically incubated at high (37.8 °C ± 0.3; Barott 1937) and constant incubation temperatures for optimal hatching success and chick quality, although some growers have begun utilizing incubators with cyclical reductions in temperatures, thereby mimicking natural fluctuations. Whether optimal incubation patterns differ between precocial and altricial species, or between galliformes and

passerines is unclear, but there is substantial variation among species in parental incubation behavior.

The primary cause of reduced survival in both the Low and Optimal treatments was caused by depredation of all nest contents (i.e. total clutch loss). This suggests that the experimental nestling was in some way influencing the fate of the entire nest, possibly through increased begging behavior. For example, research conducted on seabird chicks suggests that during periods of stress, such as decreased incubation temperatures, there is an increase in stress hormone (corticosterone) concentration. Increased corticosterone concentrations are linked to elevated and more aggressive begging, (Kitaysky et al. 2003), and changes in the begging behavior of one chick can impact begging behavior of all the chicks in the nest (Elderbrock, pers. Comms). Finally, there is evidence that predators can cue in on begging calls (Haskell 1994; Leech and Leonard 1997), and that nests with greater levels of begging behavior are more likely to be depredated (Leech and Leonard 1997). In this manner, changes to the experimental chick can translate into differences in total nest survival rates. More research is needed to explore the relationship between increased corticosterone concentration and nestling behavior and the potential downstream impacts on predation risk.

I also found differential survival probabilities between experimental treatments, which was driven by selective loss of Low-treatment nestlings. This reduction in survival suggests that incubation temperature can have lasting detrimental developmental effects (e.g. reduced body size, poorer body condition, and increased begging behavior) that may be too great for nestlings to overcome. In addition, I found no evidence for compensatory growth in the experimental nestlings (Table 2; Figure 4), and no evidence of an extended nestling duration for experimental individuals, suggesting that the experimental treatments resulted in smaller individuals at

fledging. Relatively smaller individuals often experience lower post-fledging survival (Magrath 1991), so it is possible that incubation temperature continued to impact mortality after they left the nest. These changes in mortality resulting from relatively small differences in incubation temperatures indicate that seemingly small perturbations to incubation can have potentially large-scale ramifications. The potential for slight changes in incubation temperature to have population-level impacts may be particularly relevant given the increasing range of disturbances experienced by birds. For example, habitat fragmentation, increased exposure to predators, and urbanization may impact incubation behavior (Crooks and Soulé 1999, Baudains and Lloyd 2007, Weston and Elgar 2007), and my results suggest that such changes could fundamentally impact the phenotype and ultimately the fitness of nestling birds. Indeed, previous research suggests that incubating females are capable of altering their incubation behavior in response to perceived predation risk (Ghalambor and Martin 2002; Fontaine and Martin 2006) and bears further investigation.

Incubation temperature plays a large role in the pre-and post-hatch development of young birds, and can have important consequences for chick survival. Reduced incubation temperatures between experimental treatments resulted in extended incubation period, smaller nestlings, lower nestling survival, and smaller fledglings leaving the nest. Although my research provides needed baseline information on the effects of incubation temperature, future research should examine additional factors, including temperature fluctuations and modifications to natural nests to influence incubation temperature. My research provides compelling evidence linking early developmental experiences such as the incubation temperature experienced by an avian embryo to nestling development, condition, and survival.

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Figures and Tables

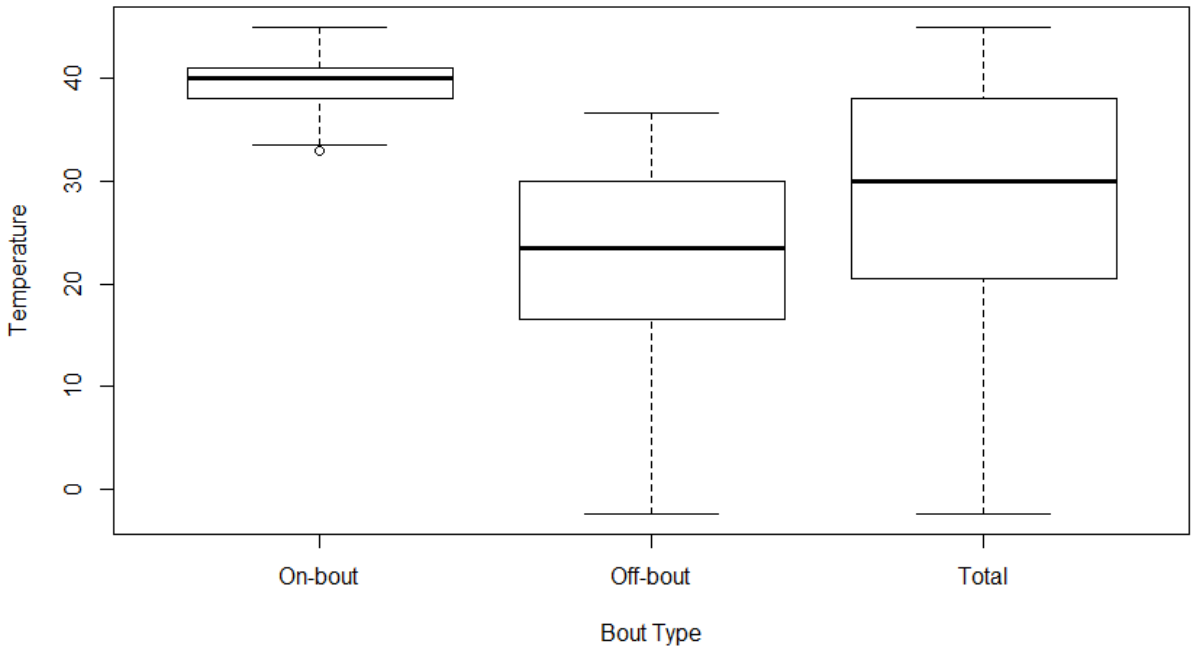

Fig. 1. Box and whisker plots of the incubation temperatures for American robin (*Turdus migratorius*) nests (n = 51) in 2015 and 2016. The horizontal line within the box indicates the median value, boundaries of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the highest and lowest values in the dataset.

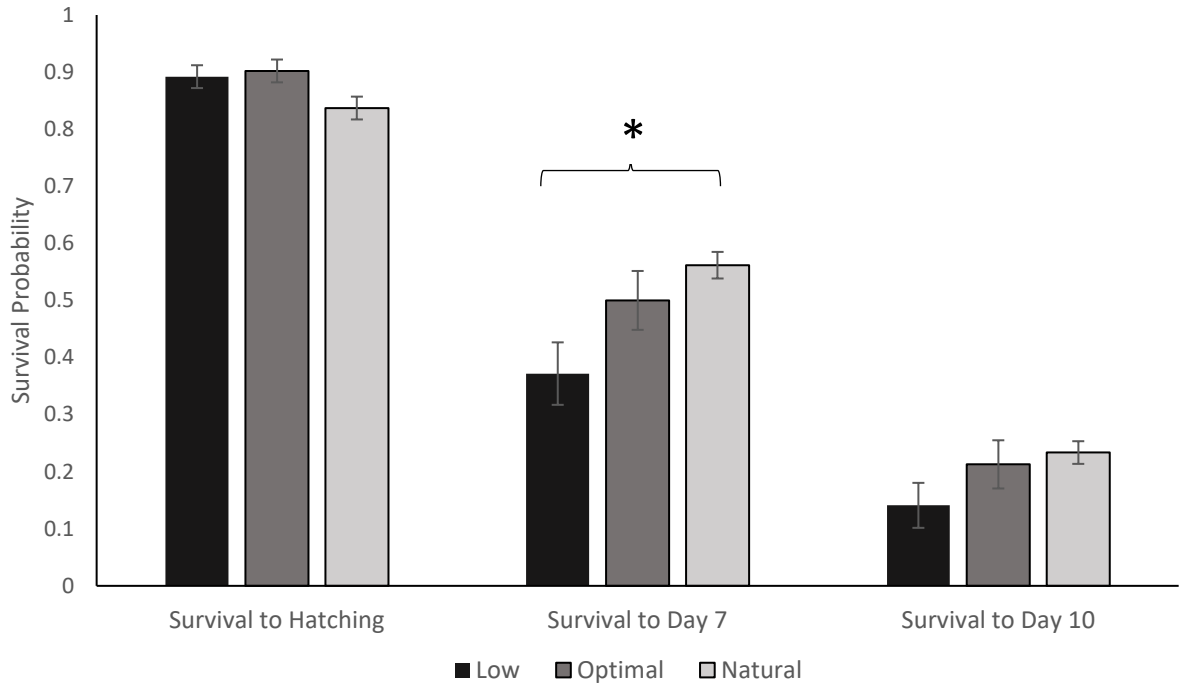

Fig. 2. Probability of American robin (*Turdus migratorius*) egg survival to hatching, nestling day 7, and nestling day 10 in two experimental incubation treatments (± SE; Low, 36.1˚C; Optimal 37.8˚C) as well as non-manipulated nests during the breeding seasons of 2015 and 2016. Hatching: Low: $n = 65$; Optimal: $n = 61$; Natural: $n = 265$; Day 7: Low: $n = 14$; Optimal: $n = 23$; Natural: $n = 148$; Day 10: Low: $n = 8$; Optimal: $n = 18$; Natural: $n = 105$. Asterisk denotes statistical significance ($p \leq 0.05$).

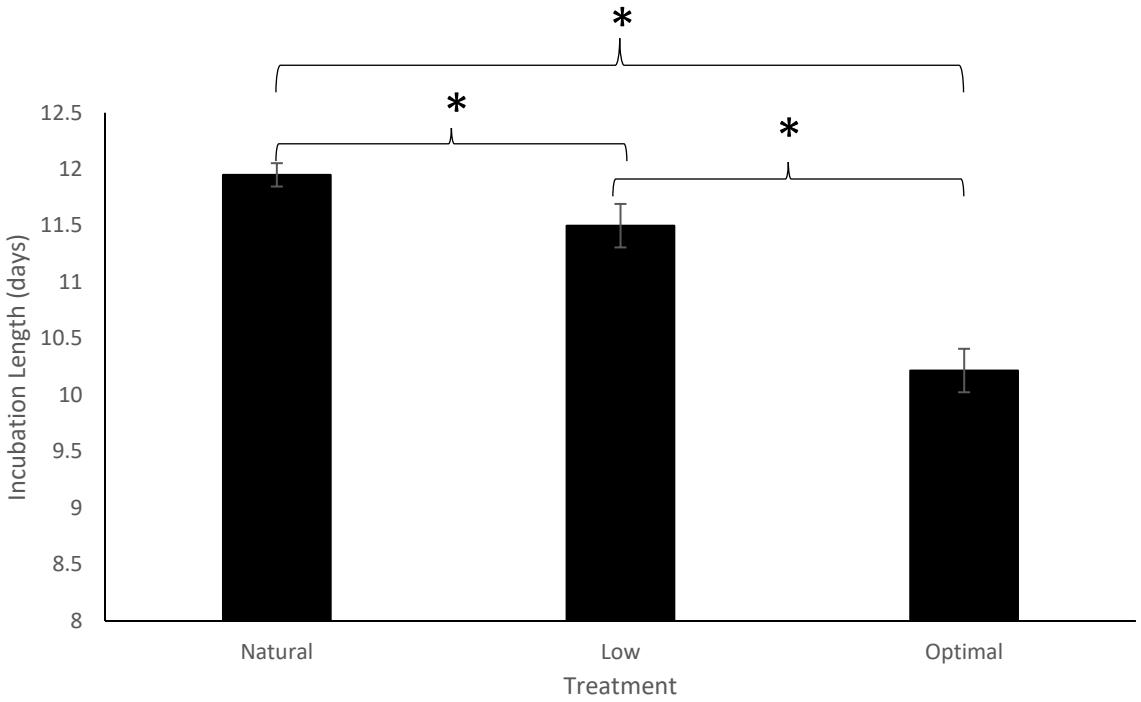

Fig. 3. Average length of incubation period (days; ± SE) for naturally incubated American robin (*Turdus migratorius*) eggs, and two experimental treatments: Low (36.1˚C) and Optimal (37.8˚C) during the 2015 and 2016 breeding seasons. Natural: $n = 265$; Low: $n = 65$; Optimal: $n = 61$. Asterisk denotes statistical significance ($p \leq 0.05$).

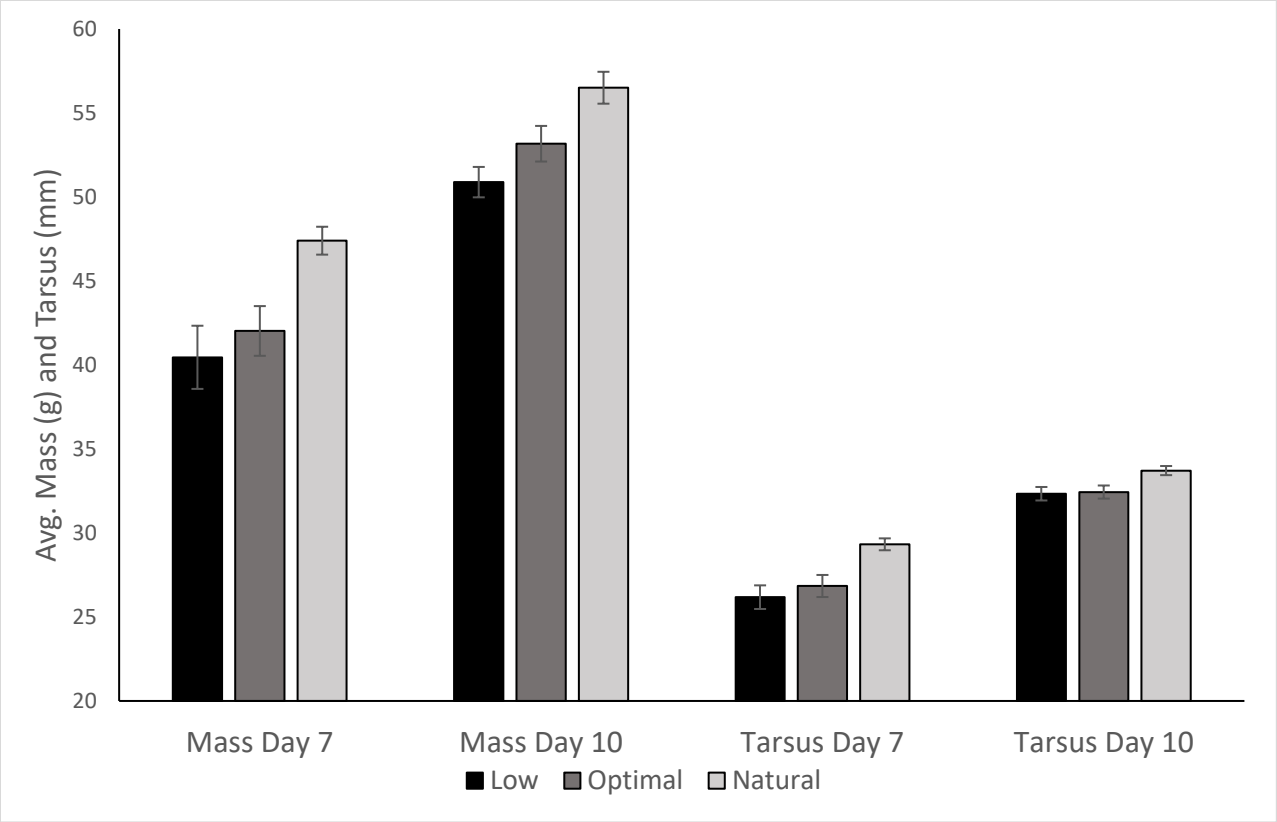

Fig. 4. Mean mass (g) and tarsus length (mm ± SE) of experimentally (Low, 36.1˚C; Optimal 37.8˚C) and naturally incubated American robin nestlings on days 7 and 10 during the nestling period of the 2015 and 2016 breeding seasons. Day 7: Low: *n* = 14; Optimal: *n* = 23; Natural: *n* = 37; Day 10: Low: *n* = 8; Optimal: *n* = 18; Natural: *n* = 26.

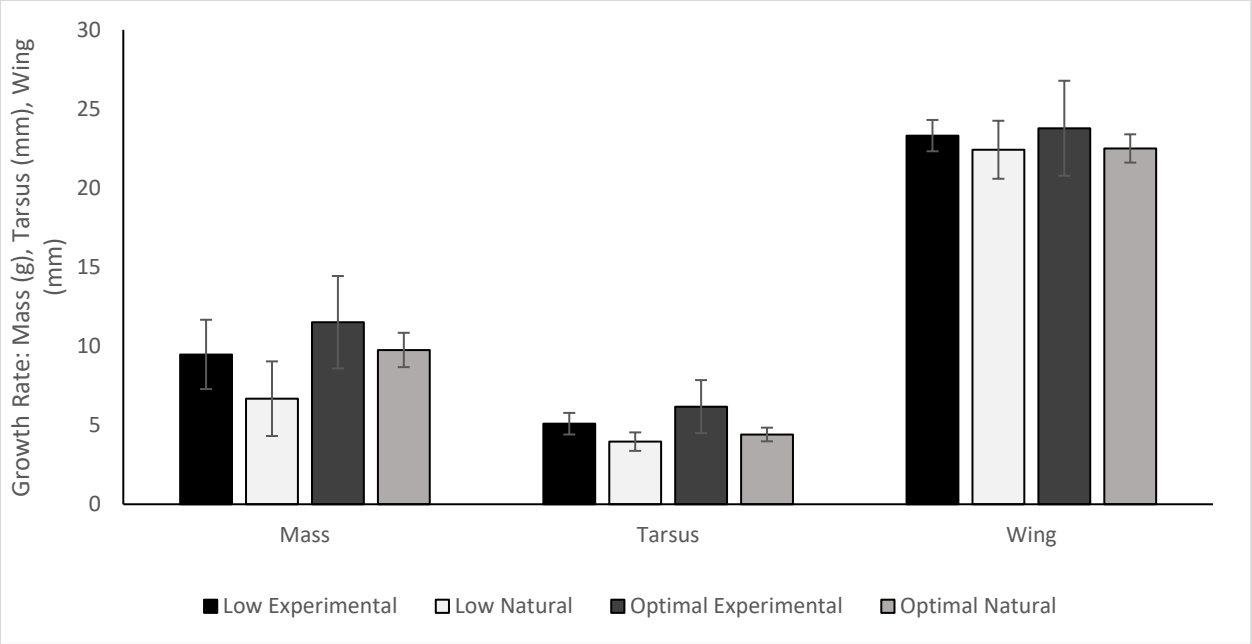

Fig. 5. Difference in mass (g), tarsus length (mm), and wing length (mm) between days 7 and 10 in nestlings from two experimental treatments (Low, 36.1˚C, black bars; Optimal, 37.8˚C, dark gray bars) and their paired naturally incubated foster nest mate (paired with Low = white, paired with Optimal = light gray, ±SE) during the 2015 and 2016 breeding seasons. Low: n = 8; Optimal: n = 18.

Table 1. Descriptive statistics for incubation patterns of American robins breeding in central Illinois, USA (n = 51). Data were collected using metallic temperature probes (iButtons). Each iButton was pre-programmed to collect data every 2 mins, removed from the nest every 2 days, and exchanged with a newly programmed iButton.

Parameter	Mean (SE)
On-bout duration (min)	33.7 (0.95)
Off-bout duration (min)	20.6 (0.40)
On-bout temperature (˚C)	39.3 (0.24)
Off-bout temperature (˚C)	22.9 (1.03)
Overall mean temperature (˚C)	28.3 (0.72)

Table 2. Results of pairwise comparisons between each focal nestling (i.e. chicks from the Low and Optimal temperature treatments) and its foster nest-mate (a naturally incubated chick) on days 7 and 10 post-hatch, and the difference between each measurement from day 7 to day 10 post-hatch (growth rate). Significant pair-wise differences bolded.

	Day 7			Day 10			Difference		
	t	df	*p*	*t*	df	*p*	*t*	df	*p*
Low - Tarsus	**-2.99**	**13**	**0.010**	-1.18	7	0.278	1.28	7	0.243
Low - Mass	**-3.00**	**13**	**0.010**	-1.2	7	0.270	0.80	7	0.452
Low - Wing	**-5.21**	**13**	**< 0.001**	**-2.84**	**7**	**0.025**	0.70	7	0.504
Optimal - Tarsus	**-3.33**	**20**	**0.003**	**-2.62**	**14**	**0.020**	-0.27	13	0.790
Optimal - Mass	**-3.09**	**20**	**0.006**	**-3.06**	**14**	**0.009**	-1.41	13	0.183
Optimal - Wing	-1.24	20	0.229	-1.2	14	0.250	-0.44	13	0.668

CHAPTER 2: INFLUENCE OF INCUBATION TEMPERATURE ON DEVELOPMENT OF THE HPA AXIS AND INNATE IMMUNE FUNCTION IN NESTLING BIRDS

Abstract

Early developmental experiences, such as incubation temperature, can have profound consequences for post-hatch physiological processes in birds. Although the effects of incubation temperature on nestling birds are still poorly understood, recent research suggests that slight differences in incubation temperature can impact survival and stress response in young birds. I examined the effects of incubation temperature on two physiological measures in nestling American robins (*Turdus migratorius*): stress response and immune function. Robin eggs were collected prior to the onset of incubation and experimentally incubated in one of two experimental treatment categories ('Low' 36.1 ˚C and 'Optimal' 37.8 ˚C). Once hatched, nestlings were fostered to a same age nest and monitored through fledging. I measured stress hormone (CORT) concentration and bacteria killing ability (BKA) on days 7 and 10 post-hatch in robin nestlings from each experimental treatment category as well as naturally incubated 'control' nestlings. 'Optimal' category individuals on day 10 post-hatch differed from 'Low' and 'Natural' category individuals with significant pre- to post-stress increase in CORT concentration. I found no effect of incubation category on BKA, but did find a pre- to post-stress reduction in BKA in a subset of incubation categories across sampling days. Finally, I found no consistent association with CORT and BKA in young robins, which may result from a stress response hyporesponsive period early in development. Robin nestlings exhibit a 'hyporesponsive' period related to CORT secretion early in development (≤ 7 days post-hatch) which may be adaptive during development via a trade-off between investing in physiological development versus somatic growth and feather development. Experimental nestlings from the

'Optimal' treatment appear to be more physiologically developed compared to individuals from the 'Low' and 'Natural' categories.

Introduction

Maternal effects are broadly defined as the non-genetic impacts females have on the expression of their offsprings' phenotype (Bernardo 1996). In many species, maternal effects are the most important environmental conditions experienced by an individual during early development (Wade 1998). Maternal effects have been documented in many taxa, influencing aspects such as offspring dispersal, anti-predator behavior, morphology, survival, and physiology (Valenzuela 2001; Meylan et al. 2002; Robert et al. 2009, DuRant et al. 2010). For placental mammals, the female's capacity for influencing her offspring's development via maternal effects is well-documented (Newth and Balls, 1979; Cowley 1991; Cheverud and Moore 1994). As a result of this intimate and prolonged association with the mother, offspring can be directly influenced by environmental factors experienced by the mother (McLaren 1981). Conditions experienced during early development have been shown to influence growth (Henry and Ulijaszek 1996), metabolism (Borges et al. 1980), immunocompetence (Mulero et al. 2007), and even sexual attractiveness in adulthood (Gustafsson et al. 1995). It is challenging, however, to experimentally manipulate the prenatal environment in mammals. In contrast, egg-laying organisms provide an ideal system for examining the impacts of the prenatal environment on development; the enclosure of all nutritional and developmental needs within and through the egg facilitating experimental manipulation (Groothuis et al. 2005).

For birds that exhibit female-only incubation behavior, incubation is a maternal effect likely to influence offspring phenotypic variability (Badyeav et al. 2003). Indeed, incubation temperatures not only influence morphological development (Hepp et al. 2006; Nord and Nilsson

2011; DuRant et al. 2013), but also development of physiological traits such as stress response (modulated by the hypothalamic-pituitary-adrenal axis; hereafter referred to as HPA axis; DuRant et al. 2010.). The HPA axis is responsible for production of glucocorticoids, which are responsible for regulating numerous physiological process (Romero 2004), and helping individuals cope with unpredictable environmental stressors (Wingfield et al. 1998; Sapolsky et al. 2000). In birds, the primary glucocorticoid hormone is corticosterone (CORT). Circulating CORT levels are typically classified as either "baseline CORT" or "stress-induced CORT." Baseline levels are thought to reflect day-to-day activity levels and metabolic demands, whereas stress-induced levels are produced in response to perceived stressors, and activate the emergency life-history stage (Wingfield et al. 1998), in which energy stores are mobilized and other physiological processes downregulated to cope with the challenge (Rensel and Schoech 2011).

The development of the HPA axis can be influenced by prenatal and early-post-natal conditions (Karrow 2006; Love et al. 2013; Merrill et al. 2015), although the manner in which early-life conditions impact HPA axis function is still challenging to predict. Prenatal experiences may influence development of the HPA axis to help prepare the organism for expected future conditions (Gluckman et al. 2005; Love and Williams 2008; Zimmer et al. 2013), potentially representing a "predictive adaptive response" (Gluckman et al. 2005).

Early-life experiences such as exposure to high levels of maternal antibodies and/or antigens are known to impact immune function in young birds for short-term antibody production and perhaps even impact long-term fitness (Merrill and Grindstaff 2014). Newly hatched birds are exposed to novel environmental antigens (Solomon 1971) and thus not capable of mounting full immune responses (Fellah et al. 2008) and therefore dependent upon maternally

transferred antibodies. However, these maternally transferred antibodies are short-lived (Grindstaff et al. 2003) and replaced by the offsprings' own functioning immune system.

The HPA axis, and more specifically CORT, influences a suite of physiological processes including bacterial killing ability (hereafter referred to as BKA), a first defense against microbial pathogens (Romero 2004; Millet et al. 2007). BKA is a functional measure of how well an individual can clear a microbial infection and thus serves as a proxy for testing the immune system. However, the relationship between CORT and BKA is complex with varying outcomes. Previous research suggests that associations between baseline CORT and BKA vary based upon life-history stage (Ellis et al. 2012; Merrill et al. 2014, Merrill et al. 2015). We have a much clearer understanding of the relationship between stress-induced CORT and BKA in which BKA decreases following a period of acute stress (Matson et al. 2006; Merrill et al. 2012; Gao et al. 2017).

Incubation temperature is known to influence CORT concentration (DuRant et al. 2010) and BKA (Ardia et al. 2010; DuRant et al. 2013). Past examinations of the links between incubation and physiology have been limited to lab-reared precocial species (DuRant et al. 2010; DuRant et al. 2013) and cavity nesting species (Ardia et al. 2010). Additionally, young passerine nestlings (≤3 days) do not secrete CORT in response to capture and handling (Romero et al. 1998; Sims and Holberton 2000), and may have limited capacity to defend against potential bacterial infections (Merrill and Grindstaff Unpubl data), but the ontogeny of stress response and immune system are poorly understood in wild birds. However, previous research on the Eurasian kestrel (*Falco tinnunculus*) suggests that growth rate is substantially suppressed when nestlings are exposed to increased CORT concentration (Müller et al. 2009). Altricial birds grow three to four times more rapidly than similarly sized precocial birds (Ricklefs 1979), and open-cup

nesters develop faster than cavity-nesting species (Bosque and Bosque 1995). This faster pace of life in altricial birds could lead to a greater negative impact of elevated CORT concentration. Furthermore, as open-cup nesting species are generally subject to greater levels of predation (Lack 1954; Ricklefs 1969), known to impact incubation temperature, these nestlings could be differentially impacted by deviations from optimal temperatures.

The potential impacts of incubation on the development period are especially critical given the importance of this period for subsequent survival (Warner and Andrews 2002; Jones et al. 2017). Therefore, examining the influence of maternal effects on early offspring development may improve our understanding of underlying factors driving variation in individual survival and fitness. Increased investment in immunocompetence and development of stress response may therefore result in a size reduction and/or an increase in development time, both of which could lead to increased predation risk (Martin et al. 2001). I examined the effects of incubation temperature on development of stress response and immune response in American robin (hereafter referred to as "robin(s)"; *Turdus migratorius*) nestlings incubated under experimental ('Optimal' and 'Low' temperatures) and natural conditions. I examined baseline and stress-induced levels of CORT and BKA of nestling robins at days 7 and 10 post-hatch to assess the impact of incubation temperature on, as well as to document the development of these two important physiological processes. I hypothesized that incubation temperature would impact development of stress response and BKA, but that the effect would be smaller, or non-detectable, in day 7 birds compared to day 10 birds. More specifically, I predicted that individuals experimentally incubated in the 'Optimal' temperature treatment would most closely resemble their 'natural' counterparts in terms of their physiology and that we would observe increased CORT concentration (baseline and stress-induced) as well as a reduction in BKA across all

sampled time points in the individuals incubated under 'Low' temperature conditions. Lastly, I predicted that there would be a negative association between CORT concentration and BKA across all treatments with the 'Low' treatment nestlings expressing the strongest negative relationship.

Methods

Study Site and Species

During the breeding seasons of 2015 and 2016 I conducted my research on an 8 ha mixed-coniferous tree farm southeast of Urbana, Illinois, USA (40°02′N, 88°10′W). The site consisted mainly of Eastern white pine (*Pinus strobus*), White fir (*Abies concolor*), Balsam fir (*Abies balsamea*), Fraser fir (*Abies fraseri*), Douglas fir (*Pseudotsuga menziesii*), and Scots pine (*Pinus sylvestris*) ranging in age from saplings to 16 years old. The most common bird species observed breeding on the site were northern cardinals (*Cardinalis cardinalis*), house finches (*Haemorhous mexicanus*), common grackle (*Quiscalus quiscula*), and American robins. The focal species for my study was the American Robin, which is a large (~80 g) thrush that breeds in most terrestrial habitats through much of North America. Female robins are solely responsible for building nests as well as laying and incubating the eggs. Females typically lay one egg per day, and the mean clutch size is 4 eggs (Vanderhoff et al. 2016). In this study system, robins typically initiate nesting in late March and continue nesting until late July. At my study site, robin nests were abundant (~21 nests per ha/year), easily located and accessible (i.e. under 2 m in height), and I experienced no known cases of nest abandonment due to researcher activities. Furthermore, their size makes them ideal for studies that require blood collection at multiple time points.

Egg Collection and Incubation

I located robin nests by systematically searching all trees on my study site every other day. Nests found during the laying stage were selected as focal experimental nests to ensure little to no development had occurred prior to egg collection. All other nests were monitored to serve as potential recipient nests for cross-fostering of experimental nestlings. At clutch completion, one egg per nest was randomly selected, individually marked, and transported to the lab at the University of Illinois at Urbana-Champaign for experimental incubation. I selected two experimental temperatures 36.1 ˚C (presumed suboptimal incubation temperature; Lundy 1969; hereafter 'Low treatment') and 37.8 ˚C (presumed optimal incubation temperature; Kuehler and Good 1990; hereafter 'Optimal') based upon previous research and known thresholds for viability (DuRant et al. 2010; Nord and Nilsson 2011). I randomly placed eggs into incubators corresponding to one of the two temperature treatments (36.1 ˚C and 37.8 ˚C; Turn X7, Lyons USA, Chula Vista, Ca.) both with constant relative humidity (60-65%). All incubators were closely monitored to ensure proper incubation temperature and humidity throughout this experiment. I monitored development within eggs throughout incubation using an Ovascope to ensure continued viability of the embryos (Brinsea OvaScope Egg Viewer, Titusville, FL). One to two days prior to hatching, I transferred eggs to a non-turning incubator (of the same temperature as their turning incubator) to ensure a safe hatching environment.

Nestling Sampling

Once hatched, nestlings were weighed (only in 2016), marked with a non-toxic permanent marker, and transferred to a non-natal robin nest with same-age nestlings. I cross-fostered experimentally incubated nestlings to help disentangle effects of genotype and incubation temperatures on nestling development. I monitored survival of experimental

nestlings by checking nests every other day. On day 7 post-hatch, I banded, weighed, took a blood sample, and measured the tarsus length of each experimental nestling and one randomly selected nestling from the foster nest. All sampling was repeated on day 10 post-hatch.

Stress Hormone and Bacterial Killing Ability

To quantify the influences of incubation temperature on HPA axis function and BKA, blood samples were collected by brachial venipuncture. Nestlings were bled within 3 minutes of removal from the nest; the bird was placed in a bag for 30 minutes to elicit a stress response, removed, and bled again. Blood samples were stored on ice for no more than 6 hours before separating plasma via centrifugation. Plasma samples were stored at -80 ˚C until they were used for CORT or BKA assessment. To prepare plasma for CORT Enzyme Immunoassay (hereafter referred to as EIA) kit, room temperature plasma (5 μL) was combined with 5μL of a dissociation reagent (Arbor Assays, Ann Arbor, MI) and incubated for a minimum of 5 minutes, then 490μL of Phosphate Buffered Saline (pH 7.0) was added to make a 1:100 dilution.

Plasma CORT was quantified using a corticosterone enzyme immunoassay with previously described methods (Munro and Stabenfeldt 1984; Santymire and Armstrong 2010). The corticosterone antiserum (CJM006) and horseradish peroxidase (provided by C. Munro, University of California, Davis, CA, USA) were diluted to 1:225,000 and 1:200,000, respectively. Antiserum cross-reactivities for corticosterone were: corticosterone, 100%; desoxycorticosterone, 14.25%; tetrahydrocorticosterone, 0.9%; 11-deoxycortisol. 0.03%; prednisone, < 0.01%; prednisolone, 0.07%; cortisol, 0.23%; cortisone, < 0.01%; progesterone, 2.65%; testosterone 0.64% and estradiol 17β, < 0.01% (Santymire and Armstrong 2010; Narayan et al. 2010). The EIA was validated biochemically for robins by demonstrating: 1) parallelism between binding inhibition curves of plasma dilutions and the corticosterone standard (*r* =

0.980); and 2) significant recovery (>90%) of exogenous corticosterone (1.95 - 1000 pg/well) added to plasma (1:600; $\hat{y} = 0.848x + 22.642$, $r^2 = 0.939$). Assay sensitivity was 1.95 pg/ well and intra- and inter-assay coefficients of variation were <10%.

To quantify the influence of incubation temperature on immune function, I assessed the bactericidal killing ability of blood plasma in vitro. The methods for the bacteria-killing assay were derived from Matson et al. (2006), Millet et al. (2007), and Morrison et al. (2009). In short, I added 5 µL of plasma to a combination of CO_2 – independent medium (Gibco, Invitrogen) + 4mM $_L$-glutamine (90 µL) and the *Escherichia coli* (ATCC 8739) bacterial broth (10 µL). This solution was incubated at 40˚C for 20 minutes. I pipetted 50 µL, in duplicate, onto agar plates, which were incubated at 37 ˚C overnight. Bacterial colonies were counted the next morning and compared to control plates (95 µL of phosphate-buffered saline and bacterial broth) incubated together absent plasma. Killing ability was determined by subtracting the mean number of colonies for the two plates per bird from the mean number of control colonies and dividing that by the control mean. I standardized BKA between years to account for a year effect in which BKA was significantly higher in 2016 than 2015.

Data Analyses

I examined effects of incubation temperature on stress response using a general linear mixed-model (SAS PROC MIXED; SAS Institute, Cary, NC, USA), including only individuals for which we had both pre- and post-stress data points. These data met assumptions of normality and CORT concentration was $\log_{10}$-transformed to meet assumptions of homoscedasticity. Temperature treatment, age, and stress treatment (baseline vs. stress-induced), and ordinal date, as well as all interactions among those variables were included as fixed effects in the original model. Interaction terms that were non-significant were dropped from the model in subsequent

iterations. Due to repeated sampling of individuals at two-time points and ages, as well as sampling, more than one individual per nest, both individual and nest identity were included as random variables to account for potential non-independence. Given *a priori* hypotheses that both CORT and BKA would be dependent upon treatment, I also constructed pairwise comparisons of CORT and BKA between time points and among temperature treatments using least squares means estimates. I used a general linear mixed model (SAS PROC MIXED) to examine relationships among tarsus length (to correct for body size), CORT, and BKA at baseline and stress-induced time points. The best-supported model included the following fixed effects: ordinal date, time point (pre- or post-stress), treatment (Natural, Optimal, Low), and a time point by treatment interaction and therefore was used to test my predictions.

Results

I found little evidence for an effect of incubation temperature on CORT concentration on day 7 post-hatch (baseline: $F_{1,33} = 1.32$; $p = 0.280$; stress-induced: $F_{1,33} = 0.03$; $p = 0.966$; Fig. 5), nor did I find evidence for an effect of incubation temperature on baseline CORT on day 10 post-hatch ($F_{2,21} = 0.37$; $p = 0.695$; Fig. 5). There was, however, a significant effect of incubation temperature on stress-induced CORT concentrations at day 10 post-hatch ($F_{2,21} = 4.56$; $p = 0.022$; Fig 5). I found no effect of incubation temperature on BKA on day 7 post-hatch (baseline: $F_{2,34} = 1.05$; $p = 0.360$; stress-induced: $F_{2,34} = 1.86$; $p = 0.172$; Fig. 6) or day 10 post-hatch (baseline: $F_{2,23} = 1.02$; $p = 0.376$; stress-induced: $F_{2,23} = 0.11$; $p = 0.899$; Fig. 6).

I observed a significant increase in CORT concentration pre- to post-stress only in nestlings hatching from the Optimal incubation treatment on day 10 post-hatch ($t_{51} = -3.19$; $p = 0.002$). I observed a significant reduction in BKA pre- to post-stress in both the Natural and Low incubation treatment individuals on day 7 post-hatch (Natural: $t_{76} = 3.20$; $p < 0.002$; Low: $t_{76} =$

2.50; $p = 0.014$; Fig. 6), but no change in BKA for chicks from the Optimal temperature treatment ($t_{76} = 0.36$; $p = 0.718$). On day 10 post-hatch, I found the opposite trend in which chicks from the Optimal treatment exhibited a significant reduction in BKA from pre- to post-stress ($t_{51} = 2.22$; $p = 0.031$; Fig. 6), whereas chicks from the Natural and Low categories exhibited no change from pre- to post-stress (Natural: $t_{51} = 0.12$; $p = 0.901$; Low: $t_{51} = 0.36$; $p = 0.721$, respectively).

When all treatments were combined, I found a negative but non-significant association between pre- and post-stressor CORT concentration and tarsus length for day 7 nestlings and day 10 nestlings (Table 3). Conversely, I observed a generally positive but non-significant correlation between pre- and post-stressor BKA and tarsus length for day 7 nestlings and day 10 nestlings (Table 3). Finally, I found a negative association between stress-induced CORT and BKA for day 7 nestlings when combining all treatments ($r = 0.32$, $p = 0.004$; Fig. 7b), but no other significant associations between CORT and BKA ($p \geq 0.15$; Figs. 7a, c, d).

Discussion

My findings provide evidence that variation in incubation temperature can influence subsequent development of physiological traits in nestling passerines. Slight changes in incubation temperature (~1.7˚C) were enough to affect a broad physiological trait (CORT concentration) in robin nestlings. I found some support for my prediction that individuals incubated at 'Low' temperatures would exhibit increased CORT and reduced BKA compared to 'Optimal' and 'Natural' incubated nestlings. Contrary to my predictions, I found a general lack of evidence to support a consistent relationship in either direction between levels of CORT and BKA. However, I did find a consistent reduction in BKA after an acute stressor with no such reduction pre-stress.

While previous research suggests that circulating CORT levels and BKA may be correlated (Merrill et al. 2014; McCormick et al. 2015) such a relationship may be obscured in nestling birds due to a period of hyporesponsiveness in the HPA axis (Romero 2004). This period of down-regulated HPA axis activity resembles the mammalian hyporesponsive period in which there is a reduction in responsiveness to stress stimuli (Sapolsky and Meaney 1986). It is thought that this hyporesponsive period serves to minimize long-term consequences associated with chronically elevated glucocorticoids. This phenomenon has also been documented in juvenile birds; white-crowned sparrow (*Zonotrichia leucophrys*) nestlings do not exhibit a significant stress response prior to 7 days post-hatch although the adrenal gland is capable of up-regulating CORT secretion when presented with a challenge (Wada et al. 2007). Similarly, there is evidence suggesting that although domestic chicks (*Gallus domesticus*) do have a functional HPA axis prior to hatching, newly hatched chicks did not respond to stress (Wise and Frye 1973). Given the extended development time necessary for robins (up to 16 days in the nest prior to fledging; Howell 1942), it is possible that robin nestlings sampled 7 days post-hatch were still within this hyporesponsive period. Previous research on the cooperatively breeding Florida scrub-jay (*Aphelocoma coerulescens*) found that independent young still mounted a dampened stress response until 1 year in age, presumably an adaptive mechanism to reduce the harmful effects of chronically elevated CORT (Rensel et al. 2010). In altricial species, which undergo a substantial portion of their development post-hatch, this hyporesponsive period may be adaptive and especially important for normal growth and development. For example, feather quality and growth rate are known to suffer when birds are exposed to increased levels of CORT (Jenni-Eiermann et al. 2015; Ellis et al. 2012b). In a young nestling, which depends on feathers to aid in thermoregulation, feather quality and growth rate are of great importance.

In developing altricial species, the adaptive immune system is not fully function, as such, nestlings rely on maternally transferred antibodies and innate immune function (Fellah et al. 2008). From an immunological standpoint, the post-hatching period is crucial for nestlings. Newly hatched nestlings are exposed to novel environmental antigens and no longer supplemented via maternal antibodies. Given a baseline level of killing ability derived from maternally transferred antibodies, it is possible that early immune function in nestlings is not sensitive to incubation temperature, at least in the short-term.

Although roughly equal numbers of nestlings hatched from both experimental incubators ('Low': $n = 39$; 'Optimal': $n = 47$) those incubated in the 'Low' treatment experienced increased rates of mortality compared to 'Optimal' and 'Natural' nestlings (Chapter 1). The CORT-Fitness Hypothesis posits that individuals in poor condition should have elevated levels of baseline CORT (Bonier et al. 2009). Empirical work has shown that there are cases where this relationship exists, but there are many instances in which this association does not exist, or occurs in the opposite direction (reviewed in Bonier et al. 2009). Previous research suggests that artificially increasing levels of circulating CORT increases begging, foraging behavior, and aggression and decreases growth efficiency and compromises cognitive abilities (Kitaysky et al. 2003; Loiseau et al. 2008). This increase in begging behavior could attract predators to the nest, providing an explanation for the 31% reduction in survival of nests containing a 'Low' treatment individual (Chapter 1). In adult birds, chronic stress (e.g. elevated CORT) is known to be maladaptive with fitness-related consequences, including a dampened ability to respond to acute stressors (Rich and Romero 2005; Cyr and Romero 2007) potentially impacting a bird's ability to respond to predators. However, the use of CORT to assess fitness remains understudied

regarding the juvenile life-history stage and could have profound carry-over implications for post-fledging survival and fitness (but see Schoech et al. 2011).

Differences in avian life history strategies (e.g. altricial vs precocial young) also play a role in the manifestation of the HPA axis development and stress response. Previous research in ducklings (DuRant et al. 2010) found significant effects of temperature in pre- to post-stress CORT concentrations. Precocial young are mobile from hatching and dependent on a fully functional HPA axis to aid in response to stress stimuli. However, in this study, I found a general lack of data to support that robin nestlings are indeed mounting a detectable and biologically significant stress response. However, there was a significant effect of incubation temperature on pre- to post-stress CORT concentration at day 10, in which nestlings of the 'Optimal' treatment exhibited a significant increase in CORT levels from pre- to post-stress. This heightened stress-responsiveness in day 10 Optimal chicks compared to Natural and Low treatment chicks suggests than those nestlings were more developed that both 'Natural' and 'Low' nestlings. The more advanced physiological development in the Optimal chicks could be influenced by an increased rate of development within the egg and consequently a shorter incubation period (1.73 days; Chapter 1) resulting in carry-over effects speeding up the nestling's rate of development post-hatch as well. Altricial nestlings, requiring exclusive care from their parents during the nestling period, would not require a responsive HPA axis at a young age and could instead allot resources to somatic growth and feather development that would directly aid in post-fledging survival (Jones et al. 2017).

Under natural conditions there are many factors that can influence incubation temperature including nest characteristics (e.g. location, substrate, etc.), ambient temperature, degree of environmental exposure, risk of nest predation (Conway and Martin 1999; Deeming 2002), and

anthropogenic disturbances (St. Clair et al. 2010). Because the effects of disturbance (e.g. human disturbance, environmental alteration) often manifest in an individual's physiology before they are detectable at the population level, physiological markers can be a powerful tool for bird conservation (Wikelski and Cooke 2006; Ellis et al. 2012a). As such, it is important to understand how biotic and abiotic conditions influence incubation temperature as we have shown that incubation temperature does impact the physiology of nestling robins.

To fully understand the effects of incubation temperature on nestling-bird physiology, more observational and experimental research is needed. My experimental design maintained constant temperatures within the artificial incubators throughout the entire incubation period, whereas under 'Natural' incubation conditions, embryos would experience temperature fluctuations corresponding with the on- and off-bouts taken by the parents. Although I examined several aspects of the influence of temperature on nestling physiology, much more remains to be learned about the physiological development of nestling birds. Studying natural variation in incubation temperatures would provide greater understanding of how incubation behavior impacts the development of nestling birds under real world conditions. Experimental cooling on zebra finch (*Taeniopygia guttata*) eggs suggests that periodic cooling of eggs, as would be observed under natural conditions for robin eggs, delays development and increases metabolic costs, thereby reducing egg nutrient uptake and conversion to embryonic tissue (Olson et al. 2006.) Future research regarding the impact of incubation rhythms, more specifically the fluctuation of temperature during the incubation period is needed as well as experiments involving the biotic and abiotic factors that influence incubation behavior.

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Figures and Tables:

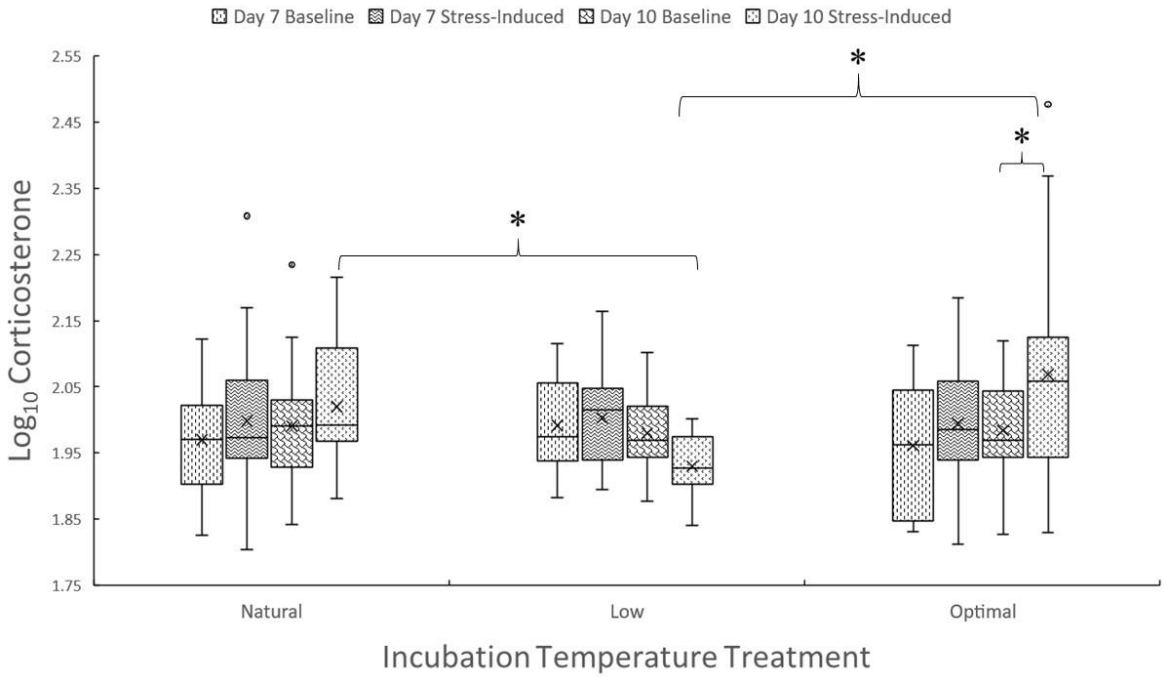

Fig. 6. Baseline and Stress-induced corticosterone concentrations in day 7 and 10 post-hatch American robin (*Turdus migratorius*) nestlings hatched from naturally and experimentally incubated eggs. Natural Day 7: $n = 41$; Natural Day 10: $n = 28$; Low Day 7: $n = 14$; Low Day 10: $n = 8$; Optimal Day 7: $n = 24$; Optimal Day 10: $n = 18$. Asterisks denotes statistical significance ($p \leq 0.01$). The horizontal line within the box indicates the median value, 'x' indicates the mean, boundaries of the box indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the maximum and minimum values in the dataset excluding outliers.

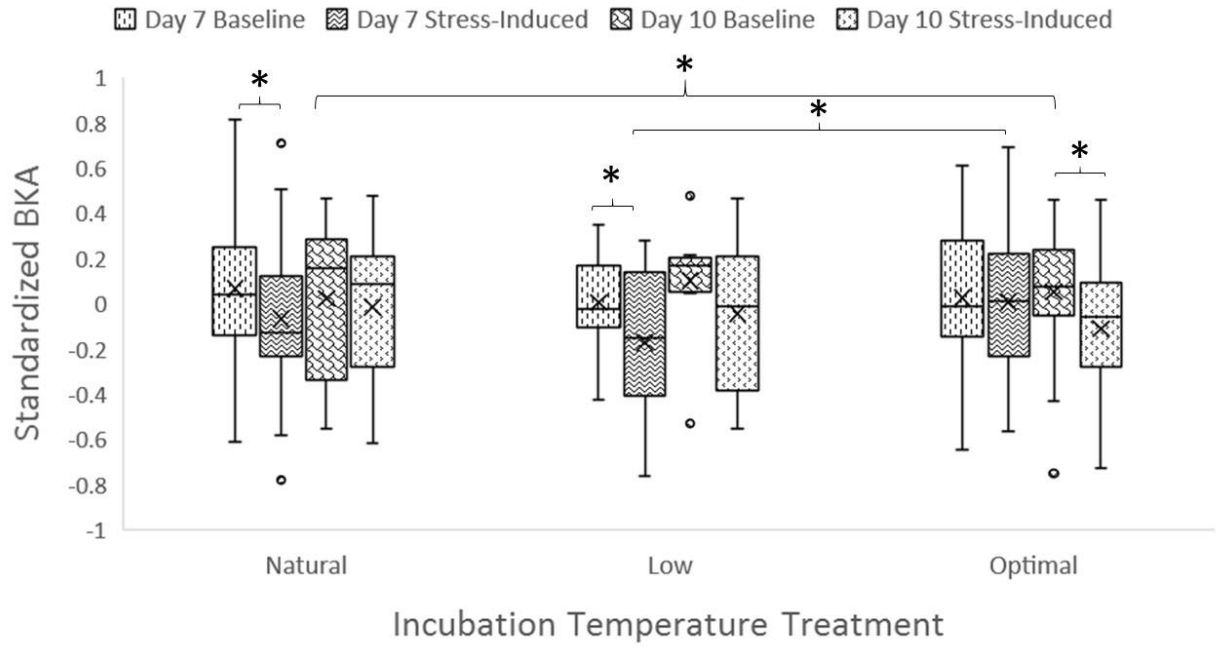

Fig. 7. Baseline and Stress-induced standardized BKA of the blood plasma in day 7 and 10 post-hatch American robin (*Turdus migratorius*) nestlings hatched from naturally and experimentally incubated eggs. Natural Day 7: $n = 41$; Natural Day 10: $n = 28$; Low Day 7: $n = 14$; Low Day 10: $n = 8$; Optimal Day 7: $n = 24$; Optimal Day 10: $n = 18$. Asterisks denotes statistical significance ($p \leq 0.01$). The horizontal line within the box indicates the median value, 'x' indicates the mean, boundaries of the box indicate the 25^{th} and 75^{th} percentiles, respectively, and the whiskers indicate the maximum and minimum values in the dataset excluding outliers.

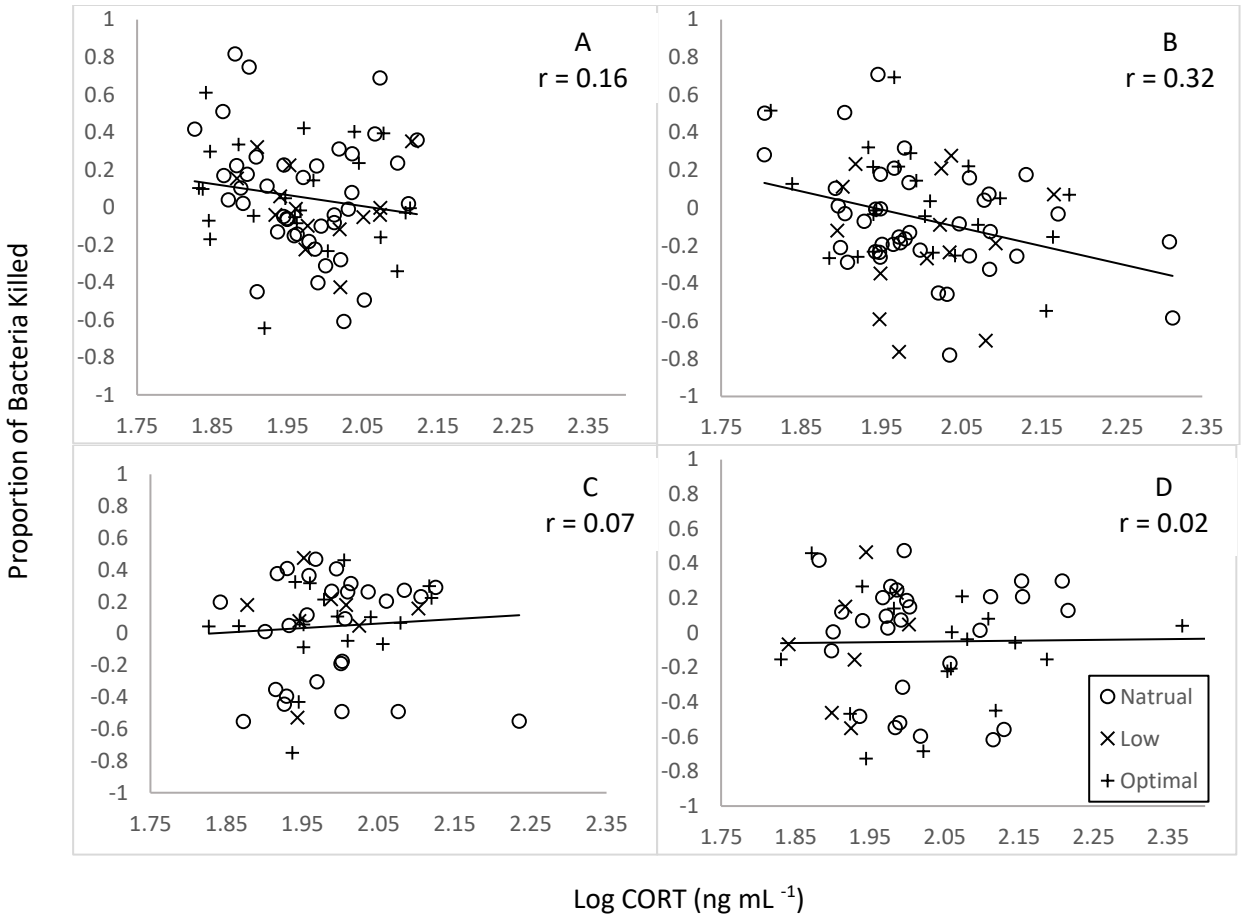

Fig. 8. Relationship between log corticosterone (CORT; ng mL $^{-1}$) and bacteria killing ability against *E. coli* in American robin nestlings (Day 7: n = 79; Day 10: n = 54). A: day 7 baseline relationship (p = 0.155) B: day 7 stress-induced relationship (p = 0.005) C: day 10 baseline relationship (p = 0.608) D: day 10 stress-induced relationship (p = 0.903).

Table. 3. Associations between two physiological measures (CORT concentration and BKA) and tarsus length (mm) at baseline and stress-induced time points for days 7 and 10 post-hatch. Bolded selections denote significance ($p < 0.05$).

	Baseline			Stress-induced		
	n	*r*	*p*	*n*	*r*	*p*
Day 7 – CORT	78	0.24	0.224	78	0.13	0.723
Day 10 – CORT	54	0.18	0.661	**54**	**0.40**	**0.032**
Day 7 – BKA	78	0.28	0.103	78	0.27	0.131
Day 10 – BKA	54	0.16	0.716	54	0.14	0.795

SUMMARY

My research focused on the effects of early developmental conditions, specifically the incubation period, critical to reproduction in birds. I examined the effects of incubation temperature on survival, growth, development, and physiology of nestling American robins (*Turdus migratorius*). From March to August of 2015 and 2016, I searched for and monitored robin nests in a mixed coniferous tree farm in central Illinois, USA. I experimentally incubated focal robin eggs at two different temperatures (36.1 and 37.8 ˚C) and fostered them to non-natal nests of the same age. I collected morphometric and survival data and used blood samples and subsequent physiological assays to estimate the effects of incubation temperature on robin nestlings.

I found that slight reductions in incubation temperature (~1.7˚ C) significantly impacted survival, growth, and two physiological measures in robin nestlings. Nestlings from either experimental treatment exhibited reductions in survival and were generally smaller than their non-manipulated Natural foster nest mate. My research also documented a physiological hyporesponsive period in which young nestlings (≤ 7 days post-hatch) regardless of experimental manipulation did not respond to exogenous stress stimuli by elevating corticosterone concentration, the primary stress hormone in birds. My research also documented an absence of a correlation between two physiological measures, speed of physiological development and innate immune defenses, which has been documented in adult birds. Collectively, my research adds to a growing body of literature exploring the effects of early developmental conditions and their impacts on nestling survival and development.

With increasing habitat destruction and human disturbance, understanding the far-reaching implications of incubation microclimate are of vital importance. My research indicates

that although incubation temperature is important for physiology, development, and survival, it also shows that temperature alone is not the only factor affecting nestling fitness. How adult birds balance the tradeoffs between self-maintenance and the needs of the developing embryos (through on- and off-bouts), can influence the phenotype of the offspring, and potentially the fitness of both parent and offspring. Increasing human disturbance, greater variation in climate, or other stressors could differentially affect developing bird embryos potentially causing population-level effects within few generations.