Nest Microclimate Manipulation Affects Growth, Development, And Heat-Shock Protein Production In The Eastern Bluebird (Sialia Sialis)

Brooke E. Sykes

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NEST MICROCLIMATE MANIPULATION AFFECTS GROWTH, DEVELOPMENT, AND HEAT-SHOCK PROTEIN PRODUCTION IN THE EASTERN BLUEBIRD (SIALIA SIALIS)

Thesis presented in partial fulfillment for the requirements of a Master of Science degree in the Department of Biology at the University of Mississippi

Submitted by: Brooke Sykes
Department of Biology
University of Mississippi

May 2020
ABSTRACT

Climatic conditions are particularly important to breeding birds, especially as recent global change has caused a shift in the timing and length of avian breeding seasons. Nest microclimate has been shown to influence avian development and parental care, however, little work has been done to examine whether increased heat poses a cost to altricial nestlings at different stages of their development. We manipulated the temperature of eastern bluebird (Sialia sialis) nest boxes to examine whether or not nestlings exhibit a heat shock response, and a difference in growth or altered parasite loads at both their early and late developmental stages. We found that heated birds were in poorer body condition over the course of the treatment and gained less mass (i.e. had a decreased growth rate) in their early development relative to control birds. Overall, heat-shock protein expression did not differ between treatments, but it was upregulated with age within heated birds, suggesting that a protective response was mounted as the birds became more developed. Feather-degrading bacterial load of thirteen-day old nestlings was highly variable and unrelated to the growth and body condition of the birds, suggesting that proliferation is influenced by ambient conditions, not individual susceptibility to parasites. Overall, our findings reveal a cost posed by excessive heat as well as a signature of tolerance, expressed when the birds are more developed and nearer to fledging. Together with other literature on cavity-nesters, this study better informs our understanding of how vertebrate animals can respond plastically to unfavorable conditions in their rearing environment and
highlights the need for additional examination of behavior as a mediator of development and physiology.
DEDICATION

This thesis is for the birds
LIST OF ABBREVIATIONS

HSP  Heat-shock protein
CFU  Colony-forming unit
PBS  Phosphate-buffered saline
q-rtpCR  Quantitative real time polymerase chain reaction
FMA  Feather meal agar
TSA  Tryptic soy agar
D2   Two days post-hatching
D5   Five days post-hatching
D13  Thirteen days post-hatching
RNA  Ribonucleic acid
cDNA Complimentary deoxyribonucleic acid
ANOVA Analysis of variance
LMER  Linear mixed effects model
ACKNOWLEDGMENTS

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working with RNA and statistical advice. I thank Dr. Brandon Barton from Mississippi State University for allowing me to use his HOBO dataloggers to collect temperature data. I thank all the faculty and staff of University of Mississippi Biology for listening to my ideas, providing feedback, and coordinating various aspects of my work, including Matt Ward, Lance Sullivan, and Reed Creaden. I would like to extend paramount gratitude to my friends and colleagues who offered me support and guidance throughout my Master’s degree.
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I. INTRODUCTION AND BACKGROUND

Climate variability across geographic clines has produced selective pressures leading to differential thermal tolerances amongst animal species, with those that evolved under more extreme conditions often possessing a broader environmental tolerance (Addo-Bediako et al. 2000). Animals with low basal metabolic rates that evolved under low levels of climate variability are especially susceptible to the effects of temperature extremes (Sekercioglu et al., 2012). Ectotherms often experience the effects of heat very drastically, as their core bodily functions including reproduction, locomotion, and growth are directly affected by temperature. They operate under a performance curve where optimum physiological functions are bounded by upper and lower temperature extremes (Deutsch et al., 2008). Endothermic animals are also highly susceptible to temperature extremes, as they invest large amounts of energy into maintaining a homeostatic temperature optimum (Scholander et al., 1950). When conditions in a species’ home range become unfavorable, they may avoid the thermal mismatch by migrating to a different region. When migration is not possible, organisms must adapt or acclimate to the change to avoid extinction.

Species that lack thermal flexibility and are unable to migrate out of their home range or acclimate to changes in climate are threatened with extinction. Already, we see this in the tropics, where heat extremes (temperatures in the 90th percentile) are now a common occurrence and pose a cost to organisms in the form of thermal stress (Buckley and Huey, 2016). Food constraints can also prevent organisms from leaving a specific range, forcing them to deal
metabolically with high temperatures. The giant panda (*Ailuropoda melanoleuca*), for example, can only occupy a small range in China due to its selective diet of bamboo. As temperatures within their constrained geographic range are projected to rise well above their thermal tolerance, they will be put at risk for extinction, or at minimum elevated heat stress levels (Zang et al., 2017).

Organisms must possess adaptations that allow them to compensate in order to alleviate the stress induced by changes in temperature (Davis and Shaw, 2001). These adaptations can include behavioral and physiological plasticity in response to both cold and heat (Huey et al., 2012) though producing these responses can be costly. Under hot conditions, they often involve some mechanism of “dumping” heat through panting, evaporative cooling (sweating), or utilizing a surface area on the body to dissipate heat (such as large ears or a large bill) (Tattersall et al., 2009). In endotherms, when temperature is elevated, metabolic rate increases, and can leave the organism with a caloric deficit if additional food is not obtained. Thermal stress also induces a physiological response in which molecular chaperones are employed to prevent protein degradation under hot conditions (Li et al., 1992). Heat-shock proteins are a class of molecular chaperones that have functions in protein folding and refolding under oxidative stress (Bukau et al., 2006). Their function is evolutionarily conserved in all known living organisms and they have broad applications as biomarkers for stress, as their upregulation often indicates an organismal response to an environmental challenge. It has also been suggested that the mild stressors that induce heat-shock proteins (henceforth HSPs) can gradually increase tolerance to future stressors over time (Dunlap and Matsumura, 1997), though producing this type of response is not without a cost. Common eiders (*Somateria mollissima*), for example, may be at increased risk for autoimmunity when heat-shock proteins are expressed at high levels.
Breeding birds are especially sensitive to thermal changes. The timing and length of avian breeding seasons are largely dependent on climatic conditions, not only because of peak prey availability under warmer conditions, but also because of the thermal requirements for developing embryos in the egg (Visser et al., 2009). The thermal optimum for incubation is somewhat narrow (35-40°C for birds, Webb 1987), and when that range is not reached or is exceeded, egg viability decreases leading to reduced hatching success. Recent studies have also shown that embryos incubated at lower temperatures expend more energy during development and have reduced immune responses post-hatching (DuRant et al., 2011). As nestlings, altricial birds are not able to independently thermoregulate and are essentially ectothermic, relying on the body heat of the brooding parent to maintain homeostasis. This dependence persists until their feathers grow in (for eastern bluebirds, typically around 7-10 days of age). While in the nest, their growth, body condition, and immune response are directly linked to the temperature of their rearing environment (Dawson et al., 2005a; Perez et al., 2008; Salaberria et al., 2014). Factors that disrupt their development, then, have potential for long-term costs like impaired growth and reduced immunity to diseases (Ardia et al., 2010).

Ectoparasites depend on specific environmental and host conditions to reproduce and proliferate successfully. They utilize host resources to feed and reproduce, sometimes resulting in reduced host survival and breeding success (Loye and Zuk 1991; Fitze et al., 2004). Ectoparasites that frequent bird nests include parasitic flies, ticks, mites, and feather-degrading bacteria, which take advantage of the developing birds’ inability to protect themselves against parasitism or escape it by leaving the nest. Feather-degrading bacteria have been sampled on the plumage of adult birds, and past experiments performed in vitro as well as in the field show that
feather-degrading bacteria alter the structural coloration of feathers, sometimes affecting sexual signals (Shawkey et al., 2007; Shawkey, Pillai, and Hill, 2009) and correlated with changes in body condition (Gunderson, Forsyth, and Swaddle, 2009). However, bacteria proliferate under specific climatic conditions, and have been shown to reproduce optimally in warm, humid environments. Prior studies of feather bacteria have focused primarily on feather condition associated metrics of signal quality (reviewed by Gunderson, 2008), but have not yet examined the relationship between temperature and bacterial load.

To date, biological indicators for heat stress in birds have been measured using behavior, growth rate and body condition, and parasite densities, but have not yet observed the upregulation of heat-shock proteins as a response to thermal mismatch. Heat-shock proteins are normally expressed constitutively in cells, but can increase specifically in response to heat and other stressors (Sanders, 1993). Heat-shock proteins potentially have broad applications as biomarkers for stress, as they are produced by all organisms; specifically, those from the heat-shock protein group 70 (HSP70s) are evolutionarily conserved in all known living species (Rensing and Maier, 1994). They function normally to assist ribosomes in producing properly folded proteins, but under stress, protect proteins from denaturation (Dunlap and Matsumura, 1997). In birds, heat-shock protein 70 has been shown to be upregulated in response to extreme temperatures, such as seasonal peaks and prolonged periods of drought associated with heat waves (Hill et al., 2013). The costs of overexpression of heat-shock proteins are not currently known, but there is evidence of for reduced lifespan in organisms like mussels (Tomanek and Zuzow, 2010).

The possible long-term costs of heat as a stressor at the organismal level as well as the potential implications for how organisms cope with changes in ambient climate are of great
interest, as globally we are experiencing hotter temperatures each year. Already, we see effects of temperature on the development, life history, and fitness of organisms exposed to temperatures outside their thermal neutral zone (TNZ; Buckley and Huey, 2016), and it has been suggested that habitat choice is heavily selected for to provide optimal rearing temperatures to breeding animals. Nest cavities of birds and bats have been of particular interest in recent work (Amat-Valero et al., 2013), as cavities provide a stable microclimate relative to ambient conditions as well as refuge from predators. Selection of nesting cavities by avian species has been demonstrated to be driven by temperature (Ardia et al., 2009), and artificial manipulation of cavity microclimate has been performed to look for its effects on incubation, growth, and future fitness. However, no studies have currently examined possible temporal effects of heat stress on altricial nestlings. Examining the links between condition, stress, and parasitism before and after nestlings are able to self-thermoregulate would better inform us how naked young cope with heat compared to older, feathered young and provide insight to their growth and development.

The eastern bluebird (Sialia sialis) serves as a good model for avian heat stress because it is a cavity nesting species, meaning that they nest in both manmade boxes and natural cavities where microclimate can easily be monitored and manipulated. They have also been intensively studied with respect to behavior (Meek and Robertson, 1994; MacDougall-Shackleton et al., 1996; Meek et al., 1992), sexual selection (Gowaty and Wagner, 1991; Siefferman and Hill, 2005), and physiology (Davis and Guinan et al, 2014; Kozlowski and Ricklefs, 2011) and are abundant locally. Here, we investigated the consequences of elevated nest microclimate on the growth, body condition, heat-shock protein expression, and parasite load of altricial eastern bluebird nestlings. We predicted that nestlings reared under experimentally raised temperature conditions would exhibit poorer body condition, and that heated birds would express heat-shock
protein 70 at greater levels than those that received the sham treatment as its function as a protective response may allow them to better tolerate heat. We also expected differences between treated and control nests with respect to bacterial prevalence, as microorganisms proliferate differentially under different temperature conditions and often thrive in hot, humid environments, making the bodies of warmer birds a more favorable environment for bacteria to grow.
II. METHODS

Field methods and nest-heat manipulation

This experiment was conducted over the course of two consecutive breeding seasons (March through August; 2018 and 2019) at two field sites in Oxford, Mississippi: The University of Mississippi Field Station (34°25'57.9"N 89°23'25.3"W) and the Ole Miss Golf Course (34°23'26.1"N 89°31'48.2"W). Nests were monitored daily during the breeding season to determine first egg lay date as well as clutch size. We measured tarsal bone length (a body size measure) two days post-hatching using digital calipers (±1mm, Rio Grande, Albuquerque, NM), and mass (a body mass measure) using a digital scale (±0.01g, Tuff Weigh, Wycomb, UK). Using these indices to later create a metric for body condition is possible due to their linear relationship (Schulte-Hostedd et al., 2005), and is a common way to quantify individual quality in avian populations (Siefferman, Hill, and Dobson, 2005). Toenails of two-day old nestlings were uniquely clipped to identify individuals within a brood until they were large enough to be banded. Treatment was assigned at random two days post-hatching, and thermodataloggers were installed in the nest to continuously monitor both internal nest temperature and ambient nest-box temperature. HOBO loggers (Onset HOBOware, Bourne, MA) were suspended in the top left corner of each nest box in both 2018 and 2019 to collect air temperature within the box on an hourly basis (Figure 1).
Figure 1: Experimental setup of nest-heat manipulation. Nests were sliced directly below the nest cup, and a heating pad surrounded by a hardware cloth sleeve was inserted and replaced every 48 hours. Temperature monitors were inserted inside the nest cup (iButton thermochron) and suspended in the top left corner of the nest (HOBO pendant logger) to collect thermal data every hour.

UniHeat 72-hour packs (American Pioneer International, Orinda, CA) were inserted directly below the nest cup and were replaced every 48 hours to maintain a consistently elevated nest temperature. iButton thermochrons (iButtonLink, Whitewater, WI) were placed in the cup of each nest in 2019 only and programmed to record temperature every hour. Nestlings were fitted with a uniquely numbered USGS band (Permit #23563) five days post-hatching and tarsal bone and mass measurements were again collected. On day five and day thirteen, 70uL of whole blood blood was collected using heparinized microhematocrit capillary tubes (Fisher Scientific
International, Hampton, NH) and stored in 1.5mL screw-cap tubes containing 630uL RNALater Stabilization Solution (Invitrogen, Carlsbad, CA). Tubes were immediately stored in a cooler on ice. Upon arrival at the lab, blood samples were refrigerated at 4°C for 24 hours. Samples were subsequently centrifuged for three minutes at 4000rpm and excess RNALater was decanted before freezing at -80°C.

Plating and quantification of bacteria

Bacterial samples were collected 13 days post-hatching using BD CultureSwabs (Becton Dickinson, Franklin Lakes, NJ). One sample was collected from the rump area, and an additional sample was collected from feathers on the rest of the body, following the methods of Gunderson, Forsynth, and Swaddle (2009). Swabs were removed from their sterile packaging and dipped in a 1.5mL microcentrifuge tube filled with 1mL of sterile 1x Phosphate Buffered Saline (Gibco, Waltham, MA). Dipped swabs were rotated over either the rump or body (wings, breast, and head) to collect bacteria, then submerged in sterile PBS and trimmed with scissors (sterilized with 70% ethanol). Samples were kept on ice after collection for a maximum of 2 hours and then brought to the lab, where they were vortexed for 10 minutes. A standardized volume (100uL) of the swab solution was inoculated on both sterile tryptic soy agar (TSA, for all culturable bacteria) and feather-meal media (FMA, selective media for keratin metabolizers, following Sangali and Brandelli 2000; Gunderson et al. 2009). Plates were incubated at 37°C for 48 hours (TSA) or 14 days (FMA) when they were counted for colony-forming units (CFU). Plates too numerous to count were subdivided into quadrats, where a selected area of the plate (25% of its surface area) was counted and then extrapolated for the total number of colonies. Plates from a number of nests (n=24) possessed fungal contamination and were excluded from future analyses,
brining the total number of nests in bacterial analyses to n=35.

**RNA extraction and quantification of heat-shock protein 70 expression**

Real-time PCR (rt-PCR) was used to determine the expression of heat-shock protein 70 as well as β-actin, a reference gene commonly used to normalize gene expression. Total RNA was extracted from avian blood using a Qiagen RNeasy Protect Animal Blood Kit (Qiagen, Hilden, Germany) and treated with RNAse-free DNase to eliminate other nucleic acids in the solution. Total RNA concentration and sample quality was determined with a BioTek plate reader (BioTek Instruments, Winooski, VT). A standard volume (10uL) of RNA solution was reverse transcribed using the Applied Biosystems cDNA Synthesis kit (Applied Biosystems, Foster City, CA) and kept at -20°C until quantitative real-time PCR could be performed. 100ng of total cDNA was diluted to a working 10ng/uL concentration for both β-actin and HSP70 reactions. Quantitative real-time PCR was performed using specific eastern bluebird primers designed with Primer 3Web (version 4.1.0; Table 1). RT-PCR was performed in triplicate using a 72-well rotor on the Qiagen Rotor-Gene Q system, using Applied Biosystems TaqMan Fast Advanced MasterMix and a FAM labeled probe. Nuclease-free water was used as a negative control in place of no-template cDNA. Cycling conditions consisted of 20 seconds at 95°C followed by 40 cycles of: 3 seconds at 95°C and 30 seconds at 56°C. A melt curve analysis was performed at the end of cycling to determine amplification specificity. The melt analysis ramped from 50°C to 99°C increasing by 1°C each step. Cycle threshold (Ct) values were obtained from each reaction and used to create a standard curve using LinRegPCR (version 11.0, Ramakers et al., 2003). Relative quantification of HSPs was performed using the 2^-ΔΔCT method to normalize for the amount of cDNA and β-actin expression in each reaction.
<table>
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**Table 1:** Primer sequences, probes, and amplicon size (base pairs) of each gene analyzed through rt-PCR in eastern bluebird whole blood.

**Statistical Analyses**

All analyses were performed in the R Statistical Computing Environment (RStudio version 3.4.2, R Core Team, Vienna, Austria, [http://www.R-project.org/](http://www.R-project.org/)) using base R functions along with the lmerTest, stats, and dplyr packages. Data were visualized using the ggplot and ggsignif packages. To calculate the relative body condition of each bird, we first used linear regressions of mass (g) against tarsal bone length (cm) for 279 nestlings sampled at their nest in 2018 and 2019. Residuals were calculated separately between the two sampling years to eliminate annual differences in morphometric indices (Figure 2), following other multi-year avian studies (e.g. O’Brien and Dawson, 2011). Here, individuals with a positive residual body mass value are considered to be in better condition than the average bird sampled, as the relationship between body mass and body size is linear and consistently used as a metric of quality in avian literature (Ardia, 2005).
Figure 2: Linear regressions for calculation of body condition. Linear regressions of mass (in grams) against tarsal bone length (in centimeters) were used as metrics for individual condition. Residuals were fitted separately by timepoint (Day 2, Day 5, and Day 13) and by year (2018 and 2019).

Measurements from individual chicks within a family were averaged to create brood means, so that each variable was represented as a repeated measure on a nest. These means were used in all analyses to account for individual replicates amongst families. To determine the degree to which the UniHeat packs elevated nest temperature, we used separate analyses of variance (ANOVAs) to compare the effect of treatment on within-nest (iButton) temperature and within box ambient (HOBO) temperature. Only data collected during the treatment period (Day 2
to Day 13 post-hatching) was included in the models. Hourly temperature measurements were averaged to create a single, mean value for temperature during the treatment period for each nest. Maximum temperature and minimum temperature per nest were also tested in the models, but did not change the direction of the effect, so mean temperature was used in all subsequent analyses.

To determine treatment effects on body condition between timepoints, we used a linear mixed-effects model. Nest ID was included in the model as a random effect to account for repeated measures on the same nest at multiple different timepoints: Day 2, Day 5, and Day 13. Similarly, we examined treatment effects on HSP70 expression using a linear mixed-effects model. Means generated from the models are +/- the standard deviation from the mean, with an α set to 0.05 to determine significance. Post-hoc comparisons were performed on the models using the “lsmeans” R package with Tukey contrasts.

To determine treatment and temperature effects on growth rate between the “early” (Day 2 to Day 5) and “late” (Day 5 to Day 13) stage, we used an analysis of variance (ANOVA), again working with brood means to eliminate pseudoreplication from individuals. Growth rate was calculated by taking the difference of body condition between timepoints, where “early growth” represents the change in body condition between Day 2 and Day 5, and “late growth” represents the change in body condition between Day 5 and Day 13, when the birds have grown in their feathers. Due to predation events that did not allow some clutches to survive for the duration of the experiment, the number of nests included in the “late stage” growth rate was reduced (Early, n=72 nests, Late, n=59 nests; Table 3). We also used ANOVAs to analyze bacterial load on TSA and FMA, as there were not repeated measures for colony counts. We included site, hatch day, and treatment as covariates in the models to look for possible interactive relationships among
ectoparasites prevalence.

To examine whether variables other than treatment were predictors of condition, growth, HSP70 expression, and bacterial load, we performed Pearson’s product-moment correlations to test for relationships amongst factors (Table 5). If correlations were found to be significant, or trending towards significance, they were incorporated into the full linear model to observe interactions with other variables.
III. RESULTS

Success of the thermal manipulation

The heat treatment successfully elevated nest temperatures by an average of 10°C in the nest cup, and heated nests were significantly warmer than control nests for the duration of the breeding season (ANOVA, $F_{1,32}=59.73$, $p<8.22e^{-9}$). Nests that hatched earlier in the breeding season (i.e., with lower Julian hatch days) trended towards having lower temperatures overall relative to nests that hatched later in the breeding season (as ambient temperatures rose). The lowest mean temperature inside the nest cup was 22.3°C, while the highest mean temperature was 46.9°C. This is a microclimate much warmer than the one required for egg incubation (26–40.5°C; Conway and Martin 2000). The effects of the heat treatment did not extend to the ambient environment inside of the next box, as significant differences were not seen in the temperature readings collected by the pendant loggers (ANOVA, $F_{1,58}=1.569$, $p=0.215$). Air temperature inside the nest box was much lower than within-cup temperature and also showed a greater range of seasonal variability as the summer progressed, the lowest recorded measurement being 15.95°C, and the highest being 29.85°C. (Figure 3).
**Figure 3: Mean temperatures experienced by each nest.** Temperature loggers collected data for the duration of the heat treatment (Day 2 to Day 13 post-hatching). Black indicates mean temperatures from control nests, where grey indicates mean temperatures from heated nests. **3A:** Mean temperature readings collected inside the nest cup by iButton thermochron loggers for n=33 nests in 2019. **3B:** Mean temperature readings collected by HOBO pendant loggers suspended in the nest box from n=37 nests in 2018 and n=21 nests in 2019. Julian day represents the continuous count of days per year, where January 1st = 1.

_Treatment effects on body condition and growth_

The linear mixed effects model showed that treatment, as well as its interaction with timepoint, significantly predicted body condition (Table 2). Tukey contrasts illustrated that there were no differences in condition two days post-hatching (pre-treatment, df=189, t=-0.205, p=0.9767), but during the treatment, heated birds were in poorer body condition at both five days post-hatching (df = 190, t=-1.314, p=0.0002) and thirteen days post-hatching (df=193, t=-1.926, p<0.001; Figure 4).
### Table 2: Results of linear-mixed effects model on body condition, with treatment and timepoint (a repeated measure) as predictors and Nest ID (Box) included as a random effect.

|                          | Estimate | Std Error | df  | t     | Pr > |\( |t| | \) |
|--------------------------|----------|-----------|-----|-------|-------|----|----|
| Treatment                | 1.9257   | 0.3242    | 192.80 | 5.939 | p<0.001 |
| Timepoint                | 0.8099   | 0.2826    | 136.84 | 2.866 | p=0.005 |
| Treatment x Timepoint    | -1.7210  | 0.4028    | 140.72 | -4.273 | p<0.001 |
| Residuals                | 1.2665   | 1.125     | 199   |       |       |

Heated birds exhibited reduced early stage growth compared to control birds (ANOVA, \( F_{1,71}=1.33, p=0.001 \); Table 3), but this effect went away in their later growth stage (ANOVA, \( F_{1,59}=0.041, p=0.839 \); Table 3). This pattern suggests that although growth rate returned to an equivalent rate when the birds began independently thermoregulating, they did not reach the mass and body condition of control bird by the end of the experiment, i.e. there was no compensatory effect for their early stage growth lag. (Figure 5)

### Table 3: Results of ANOVAs demonstrating the relationship between treatment and growth rate, using brood means of body condition to calculate growth between timepoints.

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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatment</td>
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<td>0.18</td>
<td>0.182</td>
<td>0.041</td>
<td>0.8390</td>
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<tr>
<td>Residuals</td>
<td>5</td>
<td>258.84</td>
<td>4.387</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>9</td>
<td></td>
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</table>
Figure 4: Body condition means at each timepoint for treated and control birds. Error bars represent means plus or minus standard deviation from the mean, and the open box represents control birds, whereas the filled box represents heated birds. *** indicates a p-value <0.001, and “NS” indicates nonsignificance.
Figure 5: Differences in growth rate in early and late developmental stages for heated and sham-treated birds. Control birds are represented here by the open box, and treated birds are represented by the filled box. Early stage growth represents the difference in body condition between two days and five days post-hatching, and late stage growth is the difference between five days and thirteen days post-hatching. Error bars indicate means plus or minus standard deviation from the mean for each treatment and timepoint. ** indicates significance at a p value of <0.001.

HSP70 Expression

Heat-shock protein expression did not differ significantly between heated and control birds (linear mixed-effects model, df=53.93, t=-1.86, p=0.07), nor was there an interaction between treatment and timepoint (linear mixed-effects model, df=26.57, t=1.392, p=0.18). Multiple Pearson’s product-moment correlations showed no relationships between HSP70 expression and body condition, brood size, hatch day, or nest-cup temperature (Table 5). However, within heated birds, greater HSP70 expression was seen at the Day 13 timepoint.
When visualizing the means for HSP70 expression at these different stages, it is apparent that nestlings, regardless of treatment, express HSP70 at very low levels in their early stage. However, as the birds become more developed, gaining mass and feather-cover, they begin to up-regulate their HSP70 expression. This suggests that the birds in their late stage are more capable of producing heat-shock proteins, as the group means for that timepoint were also higher than in the earlier stage (Figure 6).
<table>
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<th>Variables</th>
<th>Correlation</th>
<th>df</th>
<th>t</th>
<th>p</th>
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<td>56</td>
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<td>0.6166</td>
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<td>52</td>
<td>-0.536</td>
<td>0.5937</td>
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<tr>
<td>Hatch Day x HSP</td>
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<td>-1.5341</td>
<td>0.1306</td>
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<td>iButton Mean x HSP</td>
<td>0.107</td>
<td>54</td>
<td>0.794</td>
<td>0.4309</td>
</tr>
</tbody>
</table>

**Table 5:** Pearson’s product moment correlations to test for relationships between variables. No variables tested were significantly correlated with heat-shock protein expression.
Figure 6: HSP70 Expression Relative to β-actin. Shown are expression brood means for heat shock protein 70, where D5 is five days post-hatching and D13 is thirteen days post-hatching. 2^{-ΔACT} represents the cycling threshold value relative to beta-actin. Here, “A” and “B” represent significantly different means, where “AB” is not significantly different from either “A” or “B.” Control birds are represented by the open boxes and heated birds are represented by the solid boxes.

Feather-degrading bacterial load

Mean bacterial loads on nestling rump and body were similar and highly correlated on both TSA (Pearson’s product-moment correlation, t = 24.46, df = 202, p < 2.2e-16) and FMA (Pearson’s product-moment correlation, t = 18.437, df = 206, p < 2.2e-16), therefore we averaged rump and body counts of colony forming units (CFU) on each media type in all
subsequent analyses. Overall feather-degrading bacterial load was highly variable and did not differ between treatment (ANOVA, $F_{1,206}=0.021$, $p=0.886$, Figure 7), or site (ANOVA, $F_{1,206}=0.018$, $p=0.89$). No other variables measured – including temperature, season, or metrics of individual quality – predicted the colony counts on either type of culture medium.

**Figure 7: Bacterial loads on two different growth media between treatments.** “NS” denotes non-significance between treatment groups on both tryptic soy agar (TSA) and feather meal agar (FMA). Open boxes represent the means of control nests +/- standard deviation from the mean. Solid boxes represent heated nests.
IV. DISCUSSION

Under elevated thermal conditions, nestlings face a physiological tradeoff between maintenance of body temperature and investment in growth. Although no heat-related fatalities were observed during our experiment, nestlings in boxes with elevated nest cup temperatures showed reduced body condition relative to sham-treated controls as well as reduced growth rate (although only in their early stage). This finding is consistent with literature on other avian species (Wolf and Walsberg, 1996; Salaberria et al., 2014; Andreasson et al., 2018), including cavity nesters with similar habitats and life histories to eastern bluebirds (see Perez et al., 2008 for nest microclimate’s influence on tree swallow - *Tachycineta bicolor* - body condition). The equilibration in growth rate that we see in their later stage may be due to multiple factors, one possibility being that the birds are better able to protect themselves as they proceed in their development. Another factor to consider is that nestlings may be positioning themselves away from the nest cup, and thus, away from the heat source as they grow larger. As the birds gain mass (as well as muscle), they are able to position themselves over a greater area of the nesting cavity, and even meet their parents at its opening to receive food (Gowaty and Plissner, *Birds of North America*). This shuffling of nestlings within the nest has recently been classified as “repositioning” behavior, and has been shown to increase with parasitism in passerines such as great tits (*Parus major*; Simon et al., 2005) and Darwin’s finches (O’Connor et al., 2010). Thus, it is not out of the question that as the nestlings grow, they may be moving away from the unfavorable temperature or increasing their bouts of repositioning as a method of alleviating
stress.

It is unlikely that parental provisioning is a mediator of decreased body condition under elevated temperatures, both here and in other studies (Catry et al., 2015). Avian incubation and brooding behavior has been shown to be plastic with nest temperature (Ardia et al., 2009, Alvarez and Barba 2014); here we would expect decreased brooding, giving the parents more time to forage and supplement their nestlings with food items. As demonstrated in studies that measured incubation bouts with thermal spikes (Londoño et al., 2008) as well as others that videotaped the nest (Amininasab et al., 2016), parents spend less time incubating warm nests, so they would presumably also spend less time brooding over warm nestlings. Time off the nest is invested into foraging efficiency (Amininasab et al., 2016). However, it does not appear that there was a provisioning increase to heated nests, as there were no compensatory effects for reduced body condition in treated birds. It has been proposed that the declines in avian body condition under elevated temperatures are related to evaporative water loss (Wolf and Walsberg, 1996; Catry et al., 2015). Small birds, specifically, can lose >5% of their body mass due to dehydration (Wolf and Walsberg, 1996). So, we suggest that early-stage nestlings -- unfeathered, immobile, and confined to the nest cup where microclimate is significantly elevated -- are in poorer body condition likely due to cooling-related behaviors like panting (du Plessis et al., 2012). It is also possible that their metabolism is raised under the heat treatment, and that they are burning calories faster than they are being provisioned (Cunningham et al., 2013). In either case, is evident here that a raised nest cup temperature is associated with poorer development in altricial cavity-nesting species.

There are carryover fitness effects associated with lower passerine fledge weights including reduced survival (Blomberg et al., 2014; Vitz and Rodewald 2011; Berntsen and Bech
A study on great tits has also found that survival was negatively correlated with both increased temperature and decreased body condition (Greño, Belda and Barba 2008). Long-term survivorship could not be analyzed in our study, as the experiment was performed over the course of only two consecutive breeding seasons, but more immediate effects of poor condition have been observed in passerine species in relation to immune function (Horak et al., 1999). We suggest that reduced body condition may impose restrictions on the birds’ activity, possibly limiting the amount they are able to perform costly behaviors such as begging or repositioning.

Because cavity nesters are confined to the box and physically unable to escape the unfavorable conditions, they must cope at an intrinsic level. We predicted that altricial nestlings exposed to elevated temperatures in their early development would express heat-shock protein 70 at higher levels, as mechanism to alleviate stressors posed by the treatment. Instead, we found that HSP70 expression did not differ between heat-treated and control nestlings. Interestingly, within heated birds, late-stage heated birds expressed heat-shock protein 70 at higher levels than early-stage heated birds. This is suggestive of a protective effect, where birds are unable to mount a response to the heat treatment when they are naked and unfeathered, but late stage birds are, and upregulate their heat-shock protein expression in response to elevated nest microclimate once they are developed enough to invest in a response.

Previous studies on heat-shock protein expression in response to heat have not been performed on nestling birds, but in some species of adult birds (poultry, see Maak et al., 2003; Wang et al., 1994; Hill et al., 2013) as well as avian embryos (Gabriel et al., 2001; Givisiez et al., 2001). Other studies on vertebrate animals including fish, rats, and a handful of terrestrial ectotherms have found that heat-shock protein expression increases under heated conditions and confers protection against heat-stress (rats, see Skidmore et al., 1995; fish, Madeira et al., 2013;
Heat-shock proteins have long been thought of as reliable biomarkers for environmental stressors in different forms (reviewed in Lewis et al., 1999), but, in many of these organisms, it is not clear whether the differences in expression seen are directly due to thermal manipulation rather than natural population level variation, as suggested by Tedeschi et al., (2016). Additional work is needed to examine the long-term benefits and consequence of heat-shock protein expression at different stages in altricial avian species. There is also evidence for differential heat-shock protein expression amongst tissue types (Salway et al., 2011), so future experiments examining thermal stress may also choose to sample from multiple tissue sources to determine whether a protective response is localized to specific areas. This would provide a more comprehensive analysis of HSP expression and eliminate possibly inflated cycling threshold (Ct) values from tissues with high expression intensity.

Feather-degrading bacteria are pervasive among many avian species, and have been of interest for their possible role in degradation of sexual signals and body condition of affected individuals (reviewed in Gunderson et al., 2008). Adult eastern bluebirds have been shown to harbor feather-degrading bacteria (Gunderson, Forsyth, and Swaddle 2009), and it is thought that FDB is environmentally transmitted amongst birds (Lucas et al., 2005). We found that feather-degrading bacteria, along with total culturable microbes from swabs, did not differ between treatments and could not be predicted by any measured factors. This suggests that their presence and prevalence are highly variable in natural environments and that the nest microclimate was not a determinate of their growth on nestling bodies. It is likely that individual nestlings acquired FDB through transmission from a feeding parent, though we did not measure adult bacterial load and therefore cannot predict the exchange of parasites from parent to offspring. In adult birds,
previous work has shown sex-specific correlates of FDB on body condition, illustrating possible individual costs of feather-degradation (Gunderson, Forsyth, and Swaddle 2009), though we did not see in nestlings, and did not measure the structural color found in their feathers as they do not molt into their colorful adult plumage (pre-basic) until two to three weeks post fledging (Birds of North America species account, Gowaty and Plissner). There were also no correlations between bacterial load and HSP expression, which we also did not expect, as HSP expression has been found to be sensitive to some types of ectoparasites (Arriero et al., 2008). Ambient conditions, rather than individuals, may be a driver of parasite prevalence in bird nests, as has been found previously with insect ectoparasites (Dawson et al., 2005b). It is unclear what costs may be incurred by nestlings from feather degradation, including those incurred via effects on plumage color during their first breeding season.

Our findings demonstrate that, consistent with other work, nest microclimate plays a large role in avian development, and that altricial birds may be better equipped to deal with stressors in their later growth stage. The cost that excessive heat poses to their growth and body condition likely has long term consequences, as lower fledge weights are associated with reduced long-term fitness (Tinbergen and Boerlijst, 1990; Naef-daezner and Nuber, 2013). Broadly, this suggests that warmer microclimate may produce birds with a lower survival probability (Linden et al., 1992; Brinkhof et al., 1997). They also incur fitness effects later on in life such as reduced immune function (Horak et al., 1999) and differential resource allocation to offspring (Whittingham and Dunn, 2000) when the nest microclimate is elevated well above the normal habitable temperature range. We also show that a heat-shock response is induced by elevated heat only in the later nesting stage, and that grow rate returns to normal in the late stage. These findings suggest that altricial birds are most susceptible to thermal extremes in their non-
feathered stage. Strategies for mitigating the costs imposed by exposure to heat could include plastic behavioral responses by the parents, such as selecting nest cavities with optimal orientation relative to the sun (Ardia, Perez, and Clotfelter, 2006), modulating incubating or brooding duration (Ardia et al., 2008), and changing the composition and insulation of their nests (Windsor et al., 2013). Overall, it appears that parental plasticity plays the largest role in mediating the temperature of nest cavities.
V. CONCLUSIONS

Here, we demonstrate that elevated nest microclimate posed a significant cost to the development of altricial nestlings, which may have long-term fitness consequences and implies that they are more susceptible to a warmer microclimate in their early development. This study provides an important insight into the consequences of elevated nest temperature at different stages of nestling development. We encourage additional investigations into nest microclimate at to further determine which factors drive the diminished growth of nestlings (including parental provisioning rates and nestling metabolism), whether or not there is a specific temperature threshold that poses costs to nestlings, as well as which other correlates of nest heat can have long-term fitness outcomes on the success and survival of altricial avian species.


Gowaty PA, Plissner JH. Eastern Bluebird. The Birds of North America (A. Poole, Ed.) Ithaca: Cornell Laboratory of Ornithology; Retrieved February 24, 2020, from The Birds of North America: https://birdsna.org/Species-Account/bna/species/easblu/


VITA

Brooke E. Sykes
Department of Biology | University of Mississippi

EDUCATION

University of Mississippi
M.S. in Biology.  
Advisor: Dr. Susan L. Balenger

Arizona State University - Barrett, the Honors College
B.S. in Biological Sciences.  
Advisor: Dr. Kevin J. McGraw

TEACHING EXPERIENCE

Graduate Teaching Assistant: University of Mississippi
Biological Sciences I (2 semesters)  
Biological Sciences II (2 semesters)  
Biology Bootcamp (2 summer intersessions)  
Genetics (1 semester)  
Cell & Molecular Biology (1 semester)

RESEARCH EXPERIENCE

University of Mississippi  
Research Technician  
Principal Investigator: Dr. Susan L. Balenger  
Research Topic: Costs of experimental infection with a bacterial pathogen in a naïve host.

Arizona State University  
Honors Thesis Undergraduate Researcher  
Principal Investigator: Dr. Kevin J. McGraw  
Research Topic: Urban and sex-specific effects on carotenoid ornaments and parasitism.

Arizona State University  
Undergraduate Research Assistant  
Principal Investigator: Dr. Clive Wynne  
Research Topic: Use of a dog radial arm maze (DRAM) to model canine learning and memory.
MANUSCRIPTS

In-prep:

Sykes BE, Balenger SL. Effects of increased nest temperature on developmental phenotype of nestling eastern bluebirds (*Sialia sialis*).

PRESENTATIONS

Talks:


Posters:


**Sykes, BE** and SL Balenger. The Effects of Nest Heat Manipulation on Development, Physiology, and Parasitism in the Eastern Bluebird (*Sialia sialis*). April 2018. University of Mississippi Field Station Science Conference. Abbeville, MS.


SCHOLARSHIPS AND AWARDS

UM Graduate Student Council Research Travel Grant. 3rd place for poster. $600 2019
University of Mississippi Graduate School Travel Award. $600 2019
Luther Knight Graduate Research Scholarship. $500 2018
Grant-in-Aid of Research, Society for Integrative and Comparative Biology. $940 2018
McRight Scholarship, University of Mississippi. $2500 2017
Arizona Board of Regents High Honors Endorsement. Tuition award. 2012-2016
President’s Scholarship. Arizona State University. Tuition award. 2012-2016
Dean’s List, College of Liberal Arts & Sciences, Arizona State University. 2016
President’s Volunteer Service Award 2011, 2012

PROFESSIONAL ACTIVITIES AND MEMBERSHIPS

American Association for the Advancement of Science 2019-2020
Biology Graduate Student Society, University of Mississippi 2017-2020
Society for Integrative and Comparative Biology 2017-2020
American Ornithological Society 2017-2018
Wilson Ornithological Society 2017-2018
Association of Women in Science 2015-2016
Women in STEM, Arizona State University 2014-2016
Society of Conservation Biology, Central Arizona Chapter 2015-2016

RESEARCH MENTORING

18 Undergraduates (Field Assistants) at the University of Mississippi 2017-2019
1 Undergraduate (Lab Assistant - Emily Hopkins) at Arizona State University 2016

OUTREACH AND SERVICE

Positions Held:

President, Biology Graduate Student Society. University of Mississippi. 2019
Elected position in a university student organization.
(Acting President, 2018. Secretary, 2017).
The Biology Graduate Student Society is a representative organization and voice of the graduate student body in the Department of Biology at the University of Mississippi. During my service I organized and facilitated seminar speaker visits, worked with the undergraduate honors society (Beta Beta Beta) to host career panels, fundraised through merchandise sales to provide grants-in-aid of research for graduate students, and worked with faculty and staff to provide professional development opportunities.
**Program Specialist, Arizona Science Center.** 2016-2017  
Staff position in informal science education.  
I designed and developed K-12 youth programming within Next Generation Science Standards for use in public, private, and charter schools throughout Arizona. I conducted recurring outreach in hospitals, clinics, and community centers in low-income communities. I also led on-site programs and workshops.

**Senior Guide, Phoenix Zoo.** 2016-2017  
Staff position in informal naturalist education.  
I educated guests by engaging in discussion and interpreting exhibits. I conducted public presentations and on the train and at animal encounters.

**President, Women in STEM, Arizona State University** 2016  
Elected position in a university student organization.  
(Membership Coordinator, 2014-2015).  
Women in STEM at Arizona State University is an organization created to empower and encourage young women to pursue STEM careers. Projects included paneling at CompuGirls (an NSF funded tech workshop), tabling at campus events, fundraising, and a partnership with StateFarm to write curriculum for their annual Millennium Girls conference.

**Public Outreach:**

Upper Regional Science Fair Judge (Virtual) 2020  
International Science and Engineering Fair (Mississippi Qualifier)

Lower Elementary Science Fair Judge 2020  
Oxford Public School District

Environmental Education Tent – Hummingbird Festival 2017, 2019  
Strawberry Plains Audubon Center

Hosted Madison Palmer High School Students on a field trip 2019  
Guided tours through Biology, Physics, and Engineering at Ole Miss

Lower Elementary Science Fair Judge 2019  
Oxford Public School District

Led “Bird Bed & Breakfast” Interactive Activity for Science Day 2017  
University of Mississippi Field Station
Led an interactive activity at Conservation Science Night 2017
Phoenix Zoo

Prepared “Zoom” Lab Activity for Millennium Girls conference 2016
State Farm Millennium Girls planning committee

Participated in community cleanup day 2016
Maggie’s Place Women’s Shelter

Contributed to “Mystery Bag” Lab Activity 2015
State Farm Millennium Girls planning committee

Helped coordinate launch event 2014
*Smart Girls in the 21st Century*

Helped coordinate Womanity conference 2014
ASU Womyn’s Coalition

*University Service:*

Assisted with conference organization 2019
South Central Branch - American Society of Microbiology

Assisted at freshman welcome event for new Biology majors 2019
University of Mississippi Biology

Tabled to recruit new graduate students 2019
Association of Southeastern Biologists Meeting

Invited Guest, Tri-Beta Faculty Social 2018
University of Mississippi Biology

Co-host, BGSS/Tri-Beta Career Panel 2018
University of Mississippi Biology

Tabling, Biology Department Science Day 2018
University of Mississippi Biology

Graduate student panelist, undergrad research night 2017
University of Mississippi American Medical Women’s Association