Heat shock protein gene expression varies among tissues and populations in free-living birds.

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ABSTRACT

Climate change is dramatically altering our planet, yet our understanding of mechanisms of thermal tolerance is limited in wild birds. We characterized natural variation in heat shock protein (HSP) gene expression among tissues and populations of free-living Tree Swallows (*Tachycineta bicolor*). We focused on HSPs because they prevent cellular damage and promote recovery from heat stress. We used quantitative PCR to measure gene expression of three HSPs, including those in the HSP70 and HSP90 families that have robust experimental connections to heat in past literature. First, to evaluate how tissues and, by extension, the functions that they mediate, may vary in their thermal protection, we compared HSP gene expression among neural and peripheral tissues. We hypothesized that tissues with particularly vital functions would be more protected from heat as indicated by higher HSP gene expression. We found that brain tissues had consistently higher HSP gene expression compared to the pectoral muscle. Next, we

compared HSP gene expression across four distinct populations that span over 20 degrees of latitude (>2300 km). We hypothesized that the more southern populations would have higher HSP gene expression, suggesting greater tolerance of, or experience with, warmer local conditions. We observed largely higher HSP gene expression in more southern populations than northern populations, although this pattern was more striking at the extremes (southern Indiana vs. Alaska) and it was stronger in some brain areas than others (ventromedial telencephalon vs. hypothalamus). These results shed light on the potential mechanisms that may underlie thermal tolerance differences among populations or among tissues.

Keywords: Thermal tolerance*,* gene expression, heat shock proteins, populations, brain, ovary, muscle.

LAY SUMMARY

- Birds can be internally protected from heat by elevated production of heat shock proteins (HSPs), which prevent damage and promote recovery from heat stress.
- We characterized natural variation in heat shock protein gene expression in wild Tree Swallows (*Tachycineta bicolor*), a songbird undergoing a southward expansion in its breeding range.
- We found that the brain had consistently higher HSP gene expression compared to the flight muscle.
- We also observed higher neural HSP gene expression in more southern populations than northern populations, although this pattern was more striking at the extremes (southern Indiana vs. Alaska), and it was stronger in some brain areas than others.
- These results shed light on potential mechanisms of thermal tolerance in birds, including variation among tissues or variation among populations.

INTRODUCTION

Anthropogenic climate change is dramatically altering conditions across the globe as temperatures become increasingly hot and variable (Meehl and Tebaldi 2004; Bathiany et al. 2018). Even at sub-lethal temperatures that are common during summers in the temperate zone (35°C), heat can negatively affect body condition (Gardner et al. 2016), brain development (Shiota and Kayamura 1989), and other traits that influence survival and reproductive success (Conrey et al. 2016). While birds are able to regulate internal temperatures (Wolf and Mckechnie 2010), rising environmental temperatures may push animals to their limit of thermoregulatory capabilities (McKechnie and Wolf 2019). Understanding mechanisms of thermal tolerance is critical for wild birds as it may allow for improved predictions of population persistence. Such predictions could be instrumental in conserving the many birds that face the urgent threat of rising temperatures alongside population declines (Rosenberg et al. 2019).

While the best protection against heat stress is arguably avoidance of high temperatures, animals have evolved a range of coping mechanisms when heat is unavoidable (Etches et al. 2008; Angilletta 2009). As the next line of defense, animals initiate heat dissipation behaviors such as panting or wing spreading in birds (Etches et al. 2008; McKechnie et al. 2021), and they also shed heat via appendages, such as the bill or legs (Tattersall et al. 2017). Physiologically, birds may lessen the effect of environmental heat via facultative hyperthermia that reduces the thermal gradient between environmental and internal temperatures (Gerson et al. 2019), or they may initiate selective cooling of some critical tissues, such as the brain (Jessen 2001). However, at some point heat starts to have detrimental effects on the organism. For instance, it may negatively impact motor function (Angilletta 2009; Racinais et al. 2019), in part by slowing muscle twitch speed (Yamaguchi et al. 2010). High temperatures also may disrupt reproduction, for example, by changing odor profiles that impact mate choice or by increasing abnormalities in spermatogenesis (Fuller et al. 2019; Walsh et al. 2019). Further, heat may impair cognitive function, including song discrimination in birds (Coomes et al. 2019) and memory in both mice and humans (Lee et al. 2015; Martin et al. 2019). However, key issues still to understand are whether and how heat affects different components of the phenotype (Campos and Fedigan 2009; Angilletta 2009; de Andrade Ferrazza et al. 2017; Danner et al. 2017) including the molecular underpinnings of such variation. Strides have been made in recent years to advance the study of thermal tolerance in free-living birds from hot and dry regions (e.g. Xie et al. 2018; Smit et al. 2018; McKechnie et al. 2021), but more work is needed to expand our understanding across ecosystems.

Among the many physiological traits that facilitate thermal tolerance are heat shock proteins (HSPs). HSPs are an evolutionarily conserved response to stress – they serve to prevent cellular damage and promote recovery (Lindquist and Craig 1988; Feder and Hofmann 1999); therefore, HSP abundance may lend insight into the mechanisms of thermal tolerance. HSPs recognize non-native protein conformations and facilitate their folding, breakdown, or removal (Feder and Hofmann 1999). While there are many HSPs that respond to a range of stressors, genes in the HSP90 and HSP70 families have been specifically linked to hyperthermia (Lindquist and Craig 1988; Xie et al. 2014). Across species that have been studied so far, high HSP gene expression is associated with both direct responses to sudden heat and built-up resistance to chronic heat (Feder and Hofmann 1999; Kenkel et al. 2013; Xie et al. 2014). Therefore, cells or tissues with higher HSP mRNA or protein abundance are thought to have a higher level of protection from the negative effects of heat, compared to those with lower HSP abundance (Murugesan et al. 2017; Xie et al. 2018).

Population and tissue variation in HSP gene expression

The timing of HSP responses to heat varies among studies (Tomanek and Somero 2000; Foster et al. 2015; Wan et al. 2017), but even in captivity, baseline expression of some HSPs may track heat tolerance among populations (Fangue et al. 2006; Xie et al. 2018). At the individual level, variation in HSP expression has also been documented among tissues collected at the same moment in endotherms, including goats, rats, and chickens (Flanagan et al. 1995; Xie et al. 2014; Varasteh et al. 2015; Rout et al. 2016). Such variation suggests that some tissues - and by extension, the functions that they mediate - may be more protected from heat than others. For example, in domestic goats (*Capra aegagrus hircus*), HSP70 gene expression is higher in the liver and brain compared to the spleen and kidney (Rout et al. 2016), suggesting perhaps that metabolism, behavior, or cognition may be especially protected against heat stress. Despite potential tissue differences, HSP gene expression has been shown to be consistent within an individual or population measured two times in the same environment (Tomanek and Somero 2000; Kenkel et al. 2011), though this can vary among genes and species. The study of avian thermal tolerance, particularly HSP responses, has been greatly advanced by poultry science because heat stress is a primary economic concern in broiler and layer production (reviewed in: Etches et al. 2008; see also: Wang et al. 2013; Xie et al. 2014; Murugesan et al. 2017; Wan et al. 2017; Greene et al. 2019). For instance, HSP gene expression was higher in a breed derived from a warmer environment than a breed derived from a cooler region (Wan et al. 2017), and expression varies among tissues after both acute (24 hours) and chronic (8 weeks) heat (Xie et al. 2014). What remains unclear, is whether and how patterns of HSP abundance apply to wild, outbred birds rather than domesticated or captive subjects.

Tree Swallows are of interest to thermal physiology because of their breeding distribution across a large climatic gradient, from Alabama to Alaska (Winkler et al. 2020). This broad range has facilitated a number of key insights on latitudinal variation in life history (e.g. Dunn and Robertson 1992; Dunn et al. 2000; Stenzler et al. 2009; Akçay et al. 2016), behavior (Sellick et al. 2009; Knight et al. 2018) and physiology (reviewed in: Jones 2003; e.g. Ardia 2006; 2007; Miles et al. 2018; Zimmer et al. 2020; Winkler et al. 2020). Further, the Tree Swallow range is also interesting because while ~80% of animals are shifting their range to more northern latitudes or higher altitudes due to climate change (Root et al. 2003), the Tree Swallow breeding range is expanding to the *south* (McCaslin and Heath 2020). In the last 20 years, for example, Tree Swallows have increased in prevalence in southern Indiana, Kentucky, Tennessee and North Carolina, and in the last three decades, have begun breeding as far south as South Carolina and Alabama (Shutler et al. 2012; McCaslin and Heath 2020). Tree swallows are migratory, wintering in the Southern USA, Mexico, and Central America (Knight et al. 2018). Nevertheless, they are also philopatric, with typical natal dispersal distances of 8.38 km for females and 2.44 km for males (Winkler et al. 2005), suggesting the potential for a population to be acclimated or adapted to a particular thermal regime during the warmest summer months, despite variation in wintering habitats (Knight et al. 2018; Gow et al. 2019).

Here, we examine the pattern of natural variation in gene expression of three heat shock proteins, comparing among tissues and populations. All birds were free-living and unmanipulated, such that their HSP gene expression could integrate both plastic and evolved responses to the environment. While the processes that generate HSP variation could be influenced by sex, recent temperatures, migration length, or wintering location, distinguishing among these predictors first requires the characterization of natural differences among tissues and populations. Therefore, in this study, we focused on the pattern of HSP gene expression. We hypothesized that HSP gene expression would vary among tissues, with higher expression in

tissues that serve especially vital functions, suggesting these tissues are better protected from heat. Therefore, we predicted that HSP abundance would be higher in the brain than in the ovary or the pectoral muscle. Within the brain, we predict that the hypothalamus, which mediates many vital functions including thermoregulation (Murugesan et al. 2017), would have higher HSP gene expression compared to other brain regions. Variation in HSP gene expression among brain regions could suggest that some neural functions are more protected from heat than others. However, if we observed no difference in HSP abundance among tissues, that would suggest that protection from heat is more uniform across the body. Next, focused solely on the brain, we hypothesized that HSP gene expression would vary geographically, with individuals breeding in warmer climates having higher HSP gene expression than individuals breeding in cooler climates.

METHODS

Study Populations and Thermal Environments

We collected samples from breeding females, captured during the Spring of 2016-2018 in four different populations, shown in Figure 1: Bloomington, Indiana (IN) (39º9 N, 86º31 W, 235m elevation), Ithaca, New York (NY) (42.5ºN, 76.5ºW, 340m elevation), Burgess Junction, Wyoming (WY) (44.5ºN, 107.3ºW, 2451m elevation) and Anchorage, Alaska (AK) (61.3ºN, 149.7ºW, 40m elevation). Based on these latitudes and altitudes, we expected populations to represent a gradient of ambient temperatures. To evaluate thermal regimes in each population, we used NOAA weather data (www.ncdc.noaa.gov) to generate two key variables: First, we recorded maximum daily temperatures for the 25 years preceding this study (1991-2016) for each population. To account for missing data or offline NOAA stations, we concatenated data from the nearest NOAA station with data (distance from field site to weather station = 20.1 ± 14.3 km). We focused our analysis on breeding months in each population: May to July in Alaska and Wyoming; April to June in New York and Indiana. This 25-year spring average provides a window into the thermal environment in which these populations develop or evolve. Second, we recorded the maximum temperature the day before collection. Because the birds in this study were predominantly collected in the morning (see below), the highest temperature that the birds would have experienced recently is the maximum temperature of the day before collection.

Over the past 25 years, our four focal populations significantly differed in maximum daily temperatures during the breeding season (LMM with fixed effects of state and day of the year and population and controlling for the random effect of year. Population: $F_{3, 8796} = 2181.9$, $p < 0.0001$; Day of year: $F_{1, 8796} = 4889.9$, $p > 0.0001$; Year Wald $p = 1.00$). These 25-year values were, on average, 8°C warmer in Indiana compared to Alaska (Table 1). Similarly, the populations differed in the maximum temperature the day before collection (i.e., highest temperatures experienced recently; GLM: $F_{3, 63} = 15.71$, $p < 0.0001$). This recent temperature metric is positively correlated with 25-year average temperatures (Pearson $r = 0.29$, $p = 0.02$). It is unclear which of these (or other) temporal scales of temperature should best predict HSP variation: the day of collection, day before, previous ten days, bird's lifetime or early life experiences, or some longer scale that may reflect or drive evolutionary processes (i.e., decades or more). A full analysis is beyond the scope of this project; however, we begin to explore this question using the 25-year average maximum temperature and the maximum temperature on the day before each sample was collected. These supplementary analyses show that 25-year maximum temperatures are a better predictor of gene expression variation than the maximum

temperature on the day before each sample was collected. See Supplementary Methods and SI Tables S1-3 for details.

Field and Tissue Collection

We capitalized on already-collected samples to learn about an additional, important question in avian biology. All birds were originally collected as part of ongoing research efforts on behavioral genomics (Bentz et al. 2019) and glucocorticoid stress responses (e.g. Zimmer et al. 2020). Because we wanted to measure baseline HSP expression, our study only used unmanipulated or control birds. As a result, we have a limited sample size from populations in which researchers were testing glucocorticoid stress responses (Alaska, Wyoming, and New York).

We used similar field methods in all populations, except where noted. We captured birds in their nest boxes by hand or by using a nest box entrance trap, between 0600-1330hr. Within 3 min of capture, females were euthanized with an overdose of isoflurane, decapitated, and then the brain was dissected from the skull using sterile, RNAse-free techniques. In Indiana, we reserved additional tissues including the ovary and pectoral muscle. We excised the muscle from the right pectoralis, just distal to the midline (< 1 cm^3). We immediately froze each tissue on dry ice and then transferred to -80ºC freezer in the lab

In the lab, brains were microdissected into functional regions using clear anatomic landmarks, following (Bentz et al. 2019). Our study focused on the hypothalamus (HYPO), ventromedial telencephalon (VmT, which includes the avian medial amygdala or nucleus taeniae), and hippocampus (HPC, dissected bilaterally and pooled). We focused on these regions because they represent key behavioral centers (Goodson 2005; O'Connell and Hofmann 2012). HYPO, for example, influences the production of different hormones, such as sex steroids, corticosterone, and dopamine (Mikami 1986). HYPO also influences a range of parental, sexual, and aggressive behaviors, and it aids in thermoregulation (Murugesan et al. 2017). VmT is involved in social valence and aggression (Mikami 1986; Rosvall et al. 2012; Hong et al. 2014). HPC plays a role in spatial memory and navigation (Bingman et al. 2003; Pravosudov et al. 2006), traits that likely influence success at migrating or locating nest boxes. Thus, investigating HSP gene expression in these regions has the potential to inform how cognitive and behavioral functions may be affected by heat.

We collected all females from New York, Wyoming, and Alaska during incubation (NY n=4, WY n=4, AK n=4). In Indiana, we collected half of the females during territory establishment (n=5) and half during incubation (n=5). We found no significant difference in gene expression between breeding stages (GLM with stage as a fixed effect and individual as a random variable: all HSPs $F_{1,\ 7.36\text{-}8.21} \leq 1.17$, $p \geq 0.31$), so we pooled data by stage for analyses.

RNA Extraction and Quantitative PCR

We extracted RNA from each sample using the phenol-chloroform-based Trizol method, following the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). We then resuspended total RNA in water and analyzed quality and quantity with Epoch Microplate Spectrophotometer (Biotek, Winooski, Vermont, USA). We later treated 1 µg RNA with DNAse (Promega, Madison, Wisconsin, USA) and RNasin Ribonuclease Inhibitor (Promega, Madison, Wisconsin, USA) for reverse-transcription with oligo dT primers and Superscript III (Invitrogen, Carlsbad, California, USA).

Population and tissue variation in HSP gene expression

The resulting cDNA was used in quantitative real-time PCR (qPCR) to measure mRNA abundance of HSP90AA1, HSP90B1, and HSPA2. We focused on these HSPs because we verified that these genes were expressed in Tree Swallow transcriptomes (Bentz et al. 2019) and because they have been linked to tolerance of hyperthermia in prior experimental work (e.g. Wissing and Jäättelä 1996; Feder and Hofmann 1999; Wang et al. 2013). The HSPs in this study belong to different families: HSPA2 is in the HSP70 family, while HSP90AA1 and HSP90B1 are in the HSP90 family. As a consequence, there are regulatory differences between these families and further, between members of the same family (Stelzer et al. 2016). Members of the HSP70 family refold or break down proteins damaged by stress exposure (Lindquist and Craig 1988; Burel et al. 1992; Stelzer et al. 2016). HSPA2, is upregulated following experimental heat stress (Wang et al. 2013; Xie et al. 2014) and is also involved in steroid signaling (Ma et al. 2019) and spermatogenesis (MacPhee 2017). Members of the HSP90 family bind to steroid receptors, act as a protein transporter within the cell, and refold damaged proteins, though exact functionality varies among genes (Lindquist and Craig 1988; Burel et al. 1992; Li and Srivastava 2003; Stelzer et al. 2016). Both HSP90AA1 and HSP90B1 have been shown to be induced by experimental heat stress (Xie et al. 2014; Wan et al. 2017; Finger et al. 2018), and they serve additional cellular functions as well (Lindquist and Craig 1988; Feder and Hofmann 1999; Li and Srivastava 2003). All three candidate genes have been linked to interspecific variation in thermal tolerance in other species (Singh et al. 2014; Wan et al. 2017; Archana et al. 2017; Xie et al. 2018). Therefore, expression of these genes is likely to integrate evolved and plastic environmental response factors that influence heat tolerance mechanisms, although we cannot distinguish among these processes in this study.

We designed primers based on the Tree Swallow transcriptome (Bentz et. al., 2019) and validated them with serial dilution (efficiencies: 105.93% ± 5.13). All qPCR reactions were run on a 384 well plate in triplicate, alongside no template controls (NTCs), in a QuantStudio 5 thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using PerfeCta SYBR Green FastMix with low ROX (Quanta Biosciences, Gaithersburg, Maryland, USA). In each well we added 3 µl of cDNA diluted 1:50, or 3 µl water for NTCs, and 7 µl of mix (1.94uL water, 0.03 µl forward primer, 0.03 µl reverse primer, and 5 µl SYBR) for a 10 µl total. We set the thermocycling condition to be: 10 min at 95°C, then 40 cycles of 95°C for 30s, 60°C for 30s, and 70°C for 30s. A final dissociation phase (95°C for 1 min, 55°C for 30s, and 95°C for 30s) confirmed single-product specificity. For 7 of 84 samples, RNA concentrations were low (< 110 ng/µL) and so we modified our cDNA recipe and qPCR dilution to equalize the amount of material loaded into the qPCR reaction. Specifically, we used 400 ng of RNA for reverse transcriptase and later during qPCR, we used a 1:20 rather than 1:50 dilution qPCR to account for the lower concentration – this process closely equalized the amount of material loaded in the reaction. All samples fell within the standard curves.

We used QuantStudio Design and Analysis software v 1.5.1 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to calculate relative mRNA abundance using the comparative Ct method ($2^{-\Delta ct}$), which reports mRNA abundance for each gene of interest as the fold change in expression that is normalized to an internal reference gene. Ct values refer to the qPCR cycle number at which fluorescence exceeds background, and Ct values are therefore inversely proportional to abundance. We tested multiple reference genes, including HMBS, RPL4, and MRPS25, which have been shown to be reliable reference genes in birds (Zinzow-Kramer et al. 2014). For our first question on tissue variation in HSP gene expression, we used MRPS25 as a reference gene because it did not differ in its expression among tissues ($F_{4, 44} = 0.32$, $p = 0.86$).

Population and tissue variation in HSP gene expression

For our second question on population variation in HSP gene expression in the brain, we instead used both HMBS and PPIA as reference genes because they did not significantly differ in expression among populations ($F_{3, 60} < 1.29$ and $p > 0.29$) nor among our three brain tissues ($F_{2, 61} \leq 1.71$ and $p \geq 0.19$). In this case, the geometric mean of HMBS and PPIA was used to calculate relative gene expression ($2^{-\Delta ct}$) for the comparative Ct method. On each plate, we included intra- and inter-plate control samples (a cDNA pool derived from Tree Swallow RNA). Average intra-plate CV was 1.4% and inter-plate was 1.3%. We use standard criterion to exclude data in cases where triplicate values are too variable (i.e. >1Ct difference among replicates). This process led us to exclude two samples (one Wyoming VmT and one Indiana VmT).

Statistics

We performed statistical analyses with JMP v14 (SAS Institute, Cary, North Carolina, USA). Our total sample sizes were n=64 tissues and n=22 individuals. Total sample numbers are reflected in Table 2.

To determine our analytical approach, we first evaluated correlations in IN, where we had a larger sample size for each tissue. Within each tissue, we found some evidence of correlations among the 3 HSPs (absolute value median Pearson r = 0.65, range: 0.02 to 0.96). However, we found no strong evidence of correlations among tissues within one HSP (absolute value median Pearson r = 0.26, range: 0.03 to 0.76). In light of the functional and regulatory differences among our genes of interest (Burel et al. 1992; Stenzler et al. 2009) and the lack of published research on variation in HSP gene expression in songbirds, we used separate linear mixed models (LMM) per HSP. This approach avoids overfitting, by limiting the number of parameters per candidate model to no more than 1 per 10 observations while allowing for robust testing of our predictions regarding tissue and population variation because we had no *a priori* knowledge as to how each gene might track thermal regimes (Xie et al. 2014; Finger et al. 2018), in conserved or unique ways among tissues or among populations.

Specifically, for the question of how HSP gene expression varies among tissues, we entered tissue as a fixed effect and individual as a random effect, predicting relative gene expression ($2^{-\Delta ct}$) for each of the three HSPs. For the question of how HSP gene expression varies among populations, we included fixed effects of population, tissue, and their interaction, while controlling for the random effect of individual. Model residuals were visually inspected for normality, and this process led to a $\log_2$ transformation of gene expression data for all of models that include population. Initially, we explored potentially confounding variables that may contribute to variation across populations, including date, time since sunrise, max ambient temperatures the day before, or day of incubation. Due to limited sample sizes in this initial study characterizing HSP variation, we could not include all of these parameters in a single model. Instead, we evaluated each variable separately to determine whether to retain the variable in our final analyses. We found that HSP gene expression was unrelated to the day of the year the bird was collected ($F_{1, 0.01\text{-}0.5} \leq 2.65$, $p \geq 0.12$), amount of time since sunrise ($F_{1, 0.03\text{-}1.66} \leq 3.48$, $p \geq 0.08$), or day of incubation ($F_{1, 11\text{-}12} \leq 1.72$, $p \geq 0.21$) (Tables S5-7). Therefore, we did not retain these variables in our final models.

Because of our limited sample sizes and the potential for Type I error, we used Cook's D to evaluate our gene expression data for influential (outlier) samples. All values fall below 1, a common cutoff to indicate whether a value was potentially influential, and most fall below 0.5, a more conservative cutoff (Haubrick 2018). Across all comparisons, Cook's D = 0.05 ± 0.004,

and detailed Cook's D values are included in the Supplemental (Figure S1, S2). These data suggest that, while our sample sizes are small, our results are not driven by outlying points.

We report degrees of freedom adjusted using the Satterthwaite approximation. P-values for the fixed effects of each model were adjusted via the Benjamini-Hochberg false discovery rate. Significant pairwise comparisons (post-hoc Tukey test) are also shown in the figures. We also report marginal R^2 (R^2m: proportion of variance explained by just the fixed effects), conditional R^2 (R^2c: proportion of variance explained by both fixed and random effects), and Wald p of the random effect for our two main research questions.

RESULTS

HSP Gene Expression Across Tissues

In Indiana birds, we found a significant effect of tissue on HSP90AA1 mRNA abundance ($F_{4, 35.55} = 7.47$, adj. $p = 0.0002$, $R^2m = 0.38$, $R^2c = 0.40$; Figure 2). A post-hoc Tukey test showed that ventromedial telencephalon, hippocampus, and ovary differed significantly from pectoral muscle, though there was no difference between the hypothalamus and pectoral muscle. There was a significant effect of tissue on HSP90B1 mRNA abundance ($F_{4, 35.69} = 34.98$, adj. $p = 0.0002$, $R^2m = 0.75$, $R^2c = 0.75$); ovarian levels were higher than other tissues, and again, the ventromedial telencephalon and hippocampus were higher than the pectoral muscle. We also found a significant effect of tissue on HSPA2 mRNA abundance ($F_{4, 35.17} = 34.10$, adj. $p = 0.0002$, $R^2m = 0.73$, $R^2c = 0.74$). The Tukey test indicated higher expression in the ovary and all three neural tissues compared to pectoral muscle. Across all models, there was no significant random effect of individual (Wald $p > 0.71$), consistent with the observation that tissues vary independently from one another in their HSP gene expression.

HSP Gene Expression Across Populations

HSP gene expression in the brain also differed across populations, as detailed in Table 3 and Figure 4. In all three HSPs, we found no effect of brain region. For HSP90B1, we found a significant main effect of population. For HSP90AA1 and HSPA2, we found a population by tissue interaction. Post-hoc Tukey tests indicated several significant pairwise population differences within each tissue, which were generally more common at the extremes (i.e., Indiana vs. Alaska, see Table 3). There was no effect of individual on HSP gene expression (Wald $p > 0.22$), and the random effect of individual did not explain much variation in HSP gene expression (HSP90AA1: $R^2m = 0.47$ and $R^2c = 0.50$; HSP90B1: $R^2m = 0.41$ and $R^2c = 0.41$; HSPA2: $R^2m = 0.36$ and $R^2c = 0.36$.).

DISCUSSION

We found marked tissue- and population-level differences in HSP gene expression, despite limited sampling in some populations. Across all three HSPs studied here, most brain tissues had significantly higher HSP gene expression compared to the pectoral muscle, consistent with the view that neural functions may be especially well protected from heat. Ovarian HSP gene expression was comparably high, except for HSP90B1 which showed ovarian levels even higher than the brain. Neural HSP gene expression also varied among populations. In particular, there was a main effect of population on HSP90B1 gene expression in which Indiana was higher compared to Alaska. For HSP90AA1, populations also differed in the ventromedial

telencephalon and hippocampus, but for HSPA2, this effect was limited to the ventromedial telencephalon. Therefore, not only does the brain have consistently higher HSP gene expression than the pectoral muscle, but populations also differ in neural HSP gene expression. Furthermore, measuring one gene may not provide the full picture when asking questions about putative biomarkers of thermal tolerance. Although we cannot yet identify the processes driving these patterns, these findings in unmanipulated free-living birds nevertheless add to our understanding of the molecular framework of thermal tolerance in wild animals.

Interpreting HSP Gene Expression as a Biomarker

While animals have a plethora of behavioral and physiological responses to unavoidable heat (Etches et al. 2008; Angilletta 2009; McKechnie et al. 2021), HSP abundance is a classic thermal tolerance mechanism used to assess exposure and resistance to heat stress (Feder and Hofmann 1999). HSPs are stress-responsive and have protective qualities; thus, the leading interpretation of high HSP gene expression is that it indicates greater heat *protection* in a tissue or individual compared to those with lower HSP gene expression (Feder and Hofmann 1999; Rout et al. 2016; Murugesan et al. 2017). While less studied, a related, but not mutually exclusive interpretation of high HSP abundance is that a tissue or individual is more *sensitive* to heat (Varasteh et al. 2015; Wan et al. 2017). To the degree that such sensitivity allows for a small amount of heat to trigger a protective HSP response, this sensitivity may act as a buffer against the potentially negative effects of heat. Therefore, elevated levels could indicate higher responsiveness to heat, but most work suggests higher HSPs indicate an adaptive level of protection from heat (Feder and Hofmann 1999; Hoffmann et al. 2003; Sørensen 2010; Murugesan et al. 2017; Louis et al. 2020). However, heat-induced and baseline HSP levels are not necessarily correlated (Mezquita et al. 2001; Li et al. 2019), and there are few studies that report pre- and post-heat gene expression levels within an individual. Finally, differences in HSP abundance could indicate varying *exposure* to heat (Flanagan et al. 1995), made more complex by observations that the time-course and temperature sensitivity varies among HSP genes and species (Fangue et al. 2006; Wan et al. 2017; Finger et al. 2018; Xie et al. 2018).

The birds in our study were not experimentally exposed to heat beyond their natural environmental temperatures, which differ among populations (Table 1). Environmental temperatures can alter HSP gene expression (Foster et al. 2015; Wan et al. 2017) and species, populations, or breeds that originate from different climates may diverge in HSP expression, even when exposed to the same thermal regimes (Fangue et al. 2006; Singh et al. 2014; Wan et al. 2017; Xie et al. 2018). Thus, we cannot yet determine whether the differences seen here reflect variation in constitutive or inducible HSP gene expression. Furthermore, because the subjects in this study were free-living birds, there are many uncontrolled variables that may influence HSP gene expression, even beyond those variables that we did explore (see Supplementary Methods). In sum, we observed clear population and tissue-level variation in a breeding songbird. To the degree that gene expression patterns reflect protein abundance (Li and Biggin 2015), our results suggest that some tissues and some populations are poised to be protected from heat.

Variation Among Tissues and Genes

Variation in HSP gene expression among tissues can shed light on the functions that are more or less protected from heat. Across HSPs, most neural tissues had higher gene expression compared to the pectoral muscle, perhaps related to particularly vital neural functions that must be

protected regardless of ambient temperature. We identified region-specific expression for HSP90AA1 mRNA, which was higher in the ventromedial telencephalon compared to the hypothalamus. The functional interpretation of this result is hard to disentangle, considering that the hypothalamus and ventromedial telencephalon both contain interconnected nodes of the social behavior network (Goodson 2005; O'Connell and Hofmann 2012). Otherwise, HSP gene expression was largely similar among brain regions, suggesting that neural mediated functions such as hormone production, spatial memory, and social behavior may be, in general, well protected from heat.

We found comparatively lower levels of HSP gene expression in muscle than the brain, suggesting more limited protection from heat in the muscle (Feder and Hofmann 1999; Murugesan et al. 2017). Previous literature suggests that heat negatively impacts muscle functioning (Racinais et al. 2019; He et al. 2021). As the pectoralis is the major flight muscle (Marden 1987), its performance may be particularly relevant for swallows, who spend 80% of their day in flight (Ricklefs 1971; Rosvall 2008). Furthermore, swallow foraging (Winkler et al. 2020), migration (Gow et al. 2019), and social interactions (Rosvall et al. 2020) all rely on acrobatic flight. These flight-dependent characteristics highlight how many fitness-related traits have the potential to be affected by heat.

Some of our results varied from one gene to the next, consistent with observations that genes vary in the timing and degree of their sensitivity to heat (Feder and Hofmann 1999; Sørensen et al. 2003; Finger et al. 2018). Gene-specific results were especially evident in the ovary. In particular, HSP90B1 mRNA levels were much higher in the ovary than the brain, while HSP90AA1 and HSPA2 gene expression in the ovary was similar to levels in the brain. This gene-specific variation may be linked to the functional significance of each HSP, bearing in mind that functionality can vary within and among members of the same HSP family (Stenzler et al. 2009; Burel et al. 1992). Variation among genes also highlights the need for experimental work in wild birds, since each of these genes has been linked to heat in past work in domesticated poultry (Xie et al. 2014; Murugesan et al. 2017).

Implications of Population Variation in Neural HSP Gene Expression

Our data also showcase population variation in HSP gene expression. Females breeding in warmer climates had higher HSP gene expression compared to populations from colder climates, at least for some genes in some brain regions. While our within-population sample sizes are limited, our results mirror similar findings in other species, showing higher HSPs in populations in warmer environments in plants, ectotherms, humans, and domesticated birds (e.g. Lindquist and Craig 1988; Sørensen et al. 2003; Wan et al. 2017; Louis et al. 2020).

Although the underlying processes generating the patterns we observed will require more experimental work (e.g., a common garden), we speculate that these population differences may reflect some combination of plastic or evolved responses to different thermal environments. For example, early developmental processes may prime individuals to cope with heat (Wada 2015; Kelly 2019), and even if populations do not vary in thermal tolerance, they may simply vary in their exposure to heat (Crickenberger et al. 2015), affecting HSP levels. Notably, though, we did not find any significant differences within Indiana birds sampled during territorial establishment vs. incubation (1-2 months later), and in our population analyses, we also found no relationship with sampling date, time since sunrise, day of incubation, or maximum temperature the day before collection. Of course, populations differ in other factors that could not be quantified in this study (e.g. wintering location, timing of breeding, and ambient temperatures at various

temporal scales in the past), and our relatively limited sampling regime is not well suited to robustly testing these questions. Additionally, local climate regimes at breeding or over-wintering locations may generate different levels of HSP gene expression. Such differences in thermal tolerance have the potential to be genetic as well, as suggested by research in, for example domesticated chickens, rainbow darters, and coral (Wan et al. 2017; Oliveira et al. 2020; Louis et al. 2020). Future work is needed to tease apart these possible drivers of HSP variation among populations, including data from adult males and developing young as well.

Tree Swallows are among the first migratory species to arrive in North America in the Spring, and they breed across a broad range of environments, as far north as Alaska. Because of this, Tree Swallows are thought to be resilient to cold stress (Wang and Beissinger 2009). Our among-population results on HSP gene expression extend this idea into heat tolerance. Notably, Tree Swallows are expanding their breeding range in the American southeast (Shutler et al. 2012; McCaslin and Heath 2020), though our study did not reach the most southern portion of the range. While there may be other, non-heat related hypotheses for the Tree Swallow's southward expansion, our HSP gene expression results suggest a potential role of heat tolerance.

Conclusion

Our data reveal both tissue and population variation in HSP gene expression in Tree Swallows. Previous work laid the foundation for how heat impacts Tree Swallow behavior and reproductive success (Ardia et al. 2009; Ardia 2013; Windsor et al. 2013). Here, we extend this line of inquiry by demonstrating standing variation in gene expression levels of multiple HSPs, suggesting that mechanisms of thermal tolerance may vary within the body and among populations. Continued experimentation should explore how HSPs respond to experimental heat within particular tissues and across populations breeding at different latitudes. Such data would have the power to reveal how geographically distant, genetically distinct individuals might adapt or acclimate to their environment in similar or contrasting ways.

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Population	25-yr average max daily temperature °C ± SD	25-yr range of daily max temperatures °C
Alaska	15.12 ± 5.97	-2.78 - 30.58
Wyoming	15.90 ± 7.25	-6.67 - 36.11
New York	19.15 ± 7.73	-4.44 - 35.56
Indiana	23.12 ± 6.36	0.56 - 40.0

Table 1: The study populations differ in maximum temperatures across the previous 25 years breeding seasons.

Population	Individuals (N)	Tissue (N)				
		Pectoral Muscle	Ovary	Hypothalamus	Hippocampus	Ventromedial Telencephalon
Alaska	4	-	-	4	4	4
Wyoming	4	-	-	4	4	3
New York	4	-	-	4	4	4
Indiana	10	10	10	10	10	9

Table 2: Sample sizes per population and per tissue.

Gene expression	Population	Tissue	Population*tissue	Tukey post-hoc test
HSP90AA1	**$F_{3, 15.45} = 12.27$, adj. p = 0.002**	$F_{2, 32.49} = 0.5$, adj. p = 0.78	**$F_{6, 32.42} = 2.92$, adj. p = 0.035**	HPC: IN > WY, AK VmT: IN, NY > AK
HSP90B1	**$F_{3, 15.09} = 13.05$, adj. p = 0.0009**	$F_{2, 32.7} = 0.04$, adj. p = 0.95	$F_{6, 32.61} = 0.84$, adj. p = 0.78	IN > AK
HSPA2	$F_{3, 16.1} = 2.33$, adj. p = 0.073	$F_{2, 33.97} = 0.05$, adj. p = 0.95	**$F_{6, 33.89} = 4.74$, adj. p = 0.008**	VmT: IN, NY, WY > AK

Table 3: Neural heat shock protein gene expression across populations. Bold text indicates significant results.

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Figure 1: Map of sampled populations of breeding Tree Swallows.

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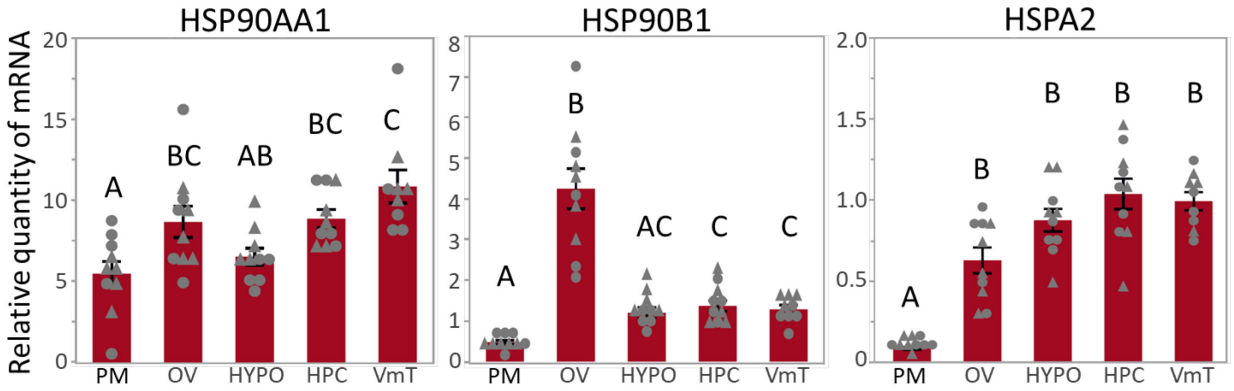

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Figure 2: Relative heat shock protein gene expression ($2^{-\Delta Ct}$) in female tree swallows breeding in southern Indiana, by tissue: pectoral muscle (PM), ovary (OV), hypothalamus (HYPO), hippocampus (HPC), ventromedial telencephalon (VmT). Triangles denote birds collected during territorial establishment, and circles denote birds collected during incubation. Gene expression is relative to reference gene MRPS25. Letters denote significant pairwise comparisons for each gene.

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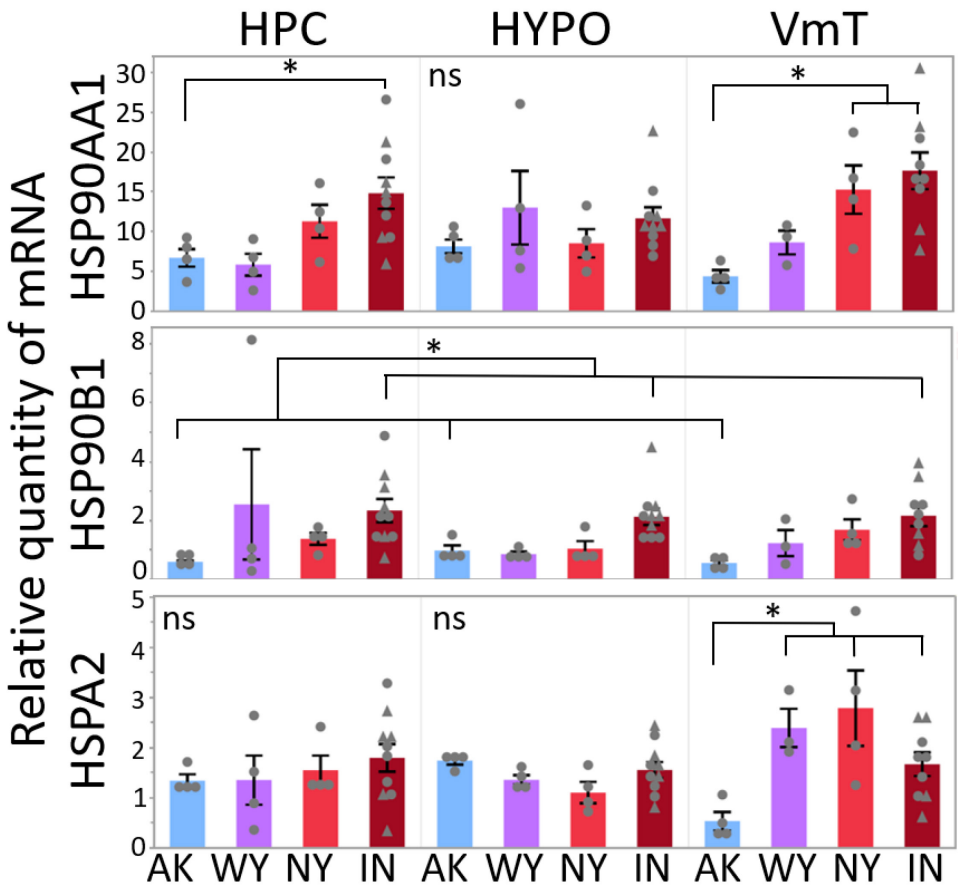

Figure 3: Relative heat shock protein gene expression ($2^{-\Delta ct}$) for hippocampus (HPC), hypothalamus (HYPO), and ventromedial telencephalon (VmT). Asterisks denote significant pairwise comparisons, including a population*tissue interaction for HSP90AA1 and HSPA2, and a main effect of population for HSP90B1. Open circles denote birds collected during territorial establishment, and closed circles denote birds collected during incubation. See Table 1 for details. Gene expression is relative to the geometric mean of reference genes HMBS and PPIA.