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RESEARCH ARTICLE

Heat alters diverse thermal tolerance mechanisms: An organismal framework for studying climate change effects in a wild bird

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Abstract

1. The ability to cope with heat is likely to influence species success amidst climate change. However, heat coping mechanisms are poorly understood in wild endotherms, which are increasingly pushed to their thermoregulatory limits.
2. We take an organismal approach to this problem, unveiling how behavioural and physiological responses may allow success in the face of sublethal heat. We experimentally elevated nest temperatures for 4 h to mimic a future climate scenario (+4.5°C) during a critical period of post-natal development in tree swallows (*Tachycineta bicolor*).
3. Heat-exposed nestlings exhibited marked changes in behaviour, including movement to cooler microclimates in the nest. They panted more and weighed less than controls at the end of the four-hour heat challenge, suggesting panting-induced water loss. Physiologically, heat induced high levels of heat shock protein (HSP) gene expression in the blood, alongside widespread transcriptional differences related to antioxidant defences, inflammation and apoptosis.
4. Critically, all nestlings survived the heat challenge, and those exposed to milder heat were *more* likely to recruit into the breeding population. Early life but sublethal heat may therefore act as a selective event, with the potential to shape population trajectories.
5. Within the population, individuals varied in their physiological response to heat, namely in HSP gene expression, which exhibited higher mean and higher variance in heat-exposed nestlings than in controls. Heat-induced HSP levels were unrelated to individual body mass, or among-nest differences in brood size, temperature, and behavioural thermoregulation. Nest identity explained a significant amount of HSP variation, yet siblings in the same nest differed by an average of ~4-fold and individuals in the population differed by as much as ~100-fold in their HSP response. This massive variation extends previous laboratory work in

model organisms showing that heat shock proteins may harbour cryptic phenotypic variation.
6. These results shed light on oft-ignored elements of thermotolerance in wild birds at a critical stage of post-natal development. By highlighting the scope of heat-induced HSP gene expression and coupling it with a suite of organismal traits, we provide a framework for future testing of the mechanisms that shape species success in the face of change.

KEYWORDS
behaviour, bird, climate change, heat responses, heat shock protein 90, individual differences

1 | INTRODUCTION

Heatwaves are intensifying (Fischer et al., 2021), and researchers are mobilizing to assess which organisms are likely to persist in the face of climate change (Moore & Schindler, 2022). As we seek to understand the mechanisms that enable persistence, organismal approaches are essential because they allow interpretation of the valence of heat effects. In response to either chronic or acute heat, animals may behaviourally change the time of day they are active (Gilbert et al., 2022), seek cooler microhabitats (Virgin & Schiel, 2023) or pant to evaporatively cool (Loughran & Wolf, 2020). Animals also may activate physiological responses, like the upregulation of heat shock proteins (HSPs) that minimize damage via protein refolding (Feder & Hofmann, 1999; Finger et al., 2018; Lindquist & Craig, 1988). HSPs can be co-regulated with other physiological mechanisms that collectively work toward restoring homeostasis (Lipshutz et al., 2022). However, these coping mechanisms may incur costs, especially as exposure to heat extends from acute to chronic. Across time scales, behavioural thermoregulation may trade-off with foraging (Hemberger et al., 2023; Mason et al., 2017) or water balance (Albright et al., 2017), and chronically elevated HSPs may have energetic costs (Feder & Hofmann, 1999). Existing data are building for each of these traits at varying durations of exposure to heat; however, we need frameworks that consider heat-response mechanisms collectively if we are to determine whether and how heat has net positive or net negative effects on the organism.

Organismal approaches to the problem of heat are especially rare in wild endotherms, despite observations that birds and mammals are increasingly pushed toward their thermoregulatory limits (Wolf & Mckechnie, 2010). Both chronic and acute heat can affect body condition (Gardner et al., 2016; Gonzalez-Rivas et al., 2020), cognition (Iyasere et al., 2021; Shiota & Kayamura, 1989), and other traits that influence lifespan and reproductive success (Conrey et al., 2016; Sisodia & Singh, 2006). Many animals spend part of their lives in ambient temperatures outside of their thermoneutral zone (Araújo et al., 2013; Buckley & Huey, 2016). As elevated ambient temperatures affect more of the temperate zone (Reidmiller et al., 2018), it is increasingly important that we understand how wild animals react.

Experimental research on intra-specific variation has the potential to play an important role in understanding these heat effects (Huey et al., 2012; Muñoz, 2022), though our understanding of the causes and consequences of *within-population* differences lags behind that of *among-population* differences (e.g. Humanes et al., 2022; Leiva et al., 2023). Such intraspecific variation has implications for a range of functionally important ecological and evolutionary issues in the face of anthropogenic change. Individual level plasticity can shape the pace of phenotypic change (Fox et al., 2019), exposing otherwise cryptic variation (Tanner et al., 2022) or shielding traits from selection (Kelly et al., 2017). HSPs, specifically, have been hypothesized to play a role in this process by potentiating phenotypic change (Jarosz & Lindquist, 2010; Rutherford & Lindquist, 1998; Tanner et al., 2022). If heritable, individual differences provide the raw material for evolution by natural selection (Lande, 1979), so populations with a high degree of standing variation should fare better in the face of climate change (Hoffmann & Sgrò, 2011).

Here we address these knowledge gaps with a short-term heat challenge that mimicked a hot afternoon we might expect under future climate change (Reidmiller et al., 2018). We focused on 12-day-old tree swallows (*Tachycineta bicolor*) confined to their nesting cavity because studying nestlings provided the opportunity for administering experimental methods in the wild. We first assessed diverse phenotypic and performance effects of heat. We focused on a set of thermoregulatory mechanisms, including (a) panting, (b) space use, (c) HSP gene expression and (d) a global analysis of other transcriptomic effects. We assessed organismal consequences by quantifying heat effects on nestling mass, begging behaviour, fledging and recruitment into the breeding population. Finally, we explored potential nest- and individual-level correlates of HSPs that may contribute to within-population variation in heat tolerance. We first hypothesized that heat would affect both nestling behaviour and physiology. We predicted that heat would increase thermoregulatory behaviours and activate diverse heat-protective physiological responses, including upregulated HSP gene expression. We further hypothesized that short-term exposure to heat during this critical period of postnatal development would have a marked effect on the organism, with carryover effects lasting beyond the short heat challenge (sensu Nord & Giroud, 2020). Exploring the valence of these effects, helps us to better understand potential adaptation to climate change.

2 | METHODS

2.1 | Study system

Our experiment occurred within nesting cavities (human-made nest boxes). Enclosed nests are thought to retain more heat (Martin et al., 2017), and we found that nest cup temperatures naturally exceed ambient by 12.3 ± 0.8°C (mean ± SE; range: 6.1–18.8°C; control data, this study). Our experiment occurred in southern Indiana, USA (39.17° N, 86.53° W) during May–June 2021, when nestlings were 12 days post-hatch ('D12'; hatch day is D1). D12 nestlings are endothermic (Marsh, 1980), and they no longer receive heat from their mother (Winkler et al., 2020). D12 nestlings have also reached asymptotic (adult-like) mass, and D12 mass is an established predictor of future fitness (Gebhardt-Henrich & Richner, 1998; McCarty, 2001). Nestlings fledge around D21 (Marsh, 1980). Nest boxes were located in full sun along the perimeter of a wetland habitat. All methods were approved by the Institutional Animal Care and Use Committee (Indiana University #21-003) and conducted with appropriate state and federal permits (Indiana Scientific Purposes #3170, USFWS Scientific Collections #MB59069B, USGS Bird Banding #23968).

2.2 | Experimental heating

We elevated nest temperatures using air-activated warmers (Uniheat 72 h, hereafter 'packs'; Albert et al., 2023; Woodruff et al., 2023) starting at midday (11:52 ± 46 min) and lasting about 4 h (4.1 ± 0.2 h). This timing exaggerates the afternoon ramp up to the heat of the day. We designed this brief, but intense heat challenge to maximize feasibility in a natural context and ensure that the heat challenge was sub-lethal. We pilot tested this design in empty nests, finding an average elevation of 5°C (see Appendix SA1 in Supporting Information). Based on previous data that occupied nests are approximately 34°C (Supporting Information, Woodruff et al., 2023), we expected experimental nest temperatures to (i) exceed the upper end of the expected thermoneutral zone (~37.5°C avg. for small songbirds, extracted from Appendix S1 in Wolf et al., 2017), and (ii) fall below expected lethal limits that can occur with prolonged exposure ≥45°C (Pollock et al., 2021). This design also minimized potential time of day effects, with all nests sampled during late afternoon when temperatures are naturally hottest.

For each heat-treated nest, we placed three packs in the box (one under the nest and one along each of two side walls, Figure S1). For each control nest, we placed three exhausted packs in this same position. We standardized nests and habituated birds to foreign objects ≥48 h in advance, and we standardized pack opening ≥0.8 h (1.5 ± 0.1 h) before the experiment to minimize inter-nest variation (see Appendix S1A). Treatments were balanced by date and brood size (average: 4.5, range: 3–6). Total sample sizes were 25 heat nests (112 nestlings) and 21 control nests (91 nestlings).

One iButton logger was secured onto the nest cup surface facing down, measuring temperature every 10 min. This iButton was along the side of the nest cup to avoid nestlings sitting directly on it. We used these data in two analyses. (1) We assessed the pace of heating by collating the 10 most recent pre-experiment iButton reads with the experimental reads. A 4-parameter logistic curve showed temperatures dramatically rising 12 min after packs were placed, after which temperatures plateaued (Figure S2). Thus, our treatment quickly elevated and sustained temperatures for the 4 h experiment. (2) We calculated mean nest and ambient temperatures, beginning when packs were placed and continuing until nestlings were sampled. Ambient data were downloaded from the National Oceanic and Atmospheric Administration (NOAA), using hourly dry bulb temperature from the nearest weather station 18.5 km away at a similar elevation (Station ID: WBAN:03893). NOAA data allowed us to control for ambient effects not already accounted for by counterbalancing by date. Mean ambient temperatures did not statistically differ between treatments ($\beta = 1.07$, SE = 3.61, $F_{1,44} = 0.77$, $p = 0.39$; Table 1, Figure 1).

A second iButton was attached to the internal box wall ~3 cm above the nest cup to measure relative humidity (RH). Mean nest RH was 54.3 ± 2.1%, and nest RH was strongly correlated with ambient RH from NOAA (Pearson $r = 0.87$; Figure S3). Minimum nest RH averaged 44.8 ± 2.1% and never reached below 25%, when evaporative cooling may be constrained (Van Dyk et al., 2019), so we did not consider RH further. Details in Table S3.

2.3 | Behavioural observations

We placed a camera (GoPro HERO Session 4) inside the nest box during pack placement. Using JWatcher (version 1.0, Blumstein & Daniel, 2007), one observer (S.N.T.) scored behaviours from the second hour of the experiment. Due to technical issues, video sample sizes included 20 heat and 17 control nests. We scored the number of nestlings visible on camera, and the number of nestlings performing each of two thermoregulatory behaviours: *Panting* was defined as >3 s of silent mouth gaping, paired with expanding and contracting body movement

TABLE 1 Treatment effects on temperature.

Temperature variable	Control	Heat
Mean nest (°C)	36.5 ± 0.6°C	40.9 ± 0.3°C
Minimum nest (°C)	34.5 ± 0.6°C	36.8 ± 0.6°C
Maximum nest (°C)	37.9 ± 0.5°C	42.7 ± 0.3°C
Mean nest elevation above ambient (°C)	12.3 ± 0.8°C	15.7 ± 0.6°C
Mean ambient (°C)	24.2 ± 1.0°C	25.2 ± 0.8°C

Note: Nest data come from iButtons, averaged per box (± standard error). Ambient data come from NOAA, collected at 1-h intervals, averaged per box, then by treatment.

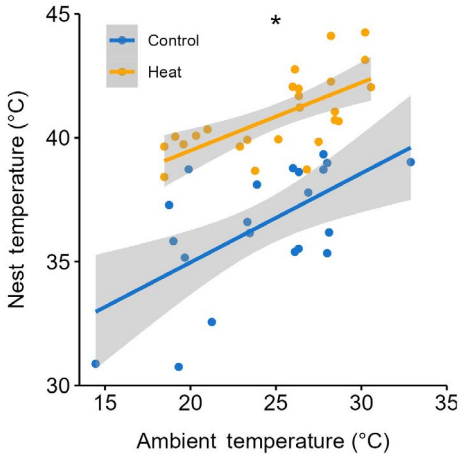

FIGURE 1 Mean nest temperature by mean ambient temperature (°C). Each point represents one nest. Heat treatment nests $n = 20$, control nests $n = 17$. Shading represents 95% confidence intervals. Asterisk indicates difference between treatments ($p < 0.05$).

(as in Woodruff et al., 2023). Panting is readily distinguished from begging because (i) panting involves rapid expanding-contracting body movements, which are uncommon during begging, and (ii) panting is typically silent while begging includes vocalizations. *Head-out-box-hole* was defined as a nestling's body along or touching the front box wall, with its head directed toward the entrance hole and its neck extended level to, or out of, the hole. This behaviour can co-occur with panting, and it is a measure of space use in which nestlings move closer to cooler ambient air near the entrance hole. Panting and head-out-box-hole data were binned into five-second intervals (max possible = 720 intervals) and scored 'present/absent' within each interval. To assess effects on other performance-related behaviours, we scored nestling begging and parental provisioning, detailed in Appendix S1B. We re-scored a subset of videos ($n = 8$), finding moderate to high repeatability for focal behaviours (intra-class correlation coefficients: 0.72–0.97); details in Appendix S1B and Table S1.

2.4 | Nestling sampling

After the heat challenge, we confirmed nestling survival (all survived, $n = 112$). We sampled blood from the largest, median, and smallest nestlings per nest, to facilitate analyses on potential mass-related correlates of heat responses. Nestlings were bled from the alar vein (~50 μL; latency from nest disturbance to blood on ice: 7:11 ± 0:15 min), except for the median mass individual, which was euthanized via overdose of isoflurane followed by rapid decapitation and bled from the trunk (latency to euthanasia: 3:17 ± 0:10 min). From euthanized nestlings, we collected additional tissues, including pectoral muscle and brain, which also express HSPs (Woodruff et al., 2022). Samples were frozen on dry ice in the field and stored at −80°C. Later, we micro-dissected brains as in (Soma et al., 2003), focusing our analyses on the hippocampus (HPC). We banded all remaining nestlings with a numbered aluminium band.

For all nestlings, we measured body mass using a digital scale (nearest 0.1 g) and flattened wing length using a stopped wing ruler (nearest 0.5 mm). Because D12 nestlings have reached asymptotic mass (McCarty, 2001), we assumed any treatment differences in mass would relate to food intake or water balance during the trial. Wing length, on the other hand, is a better measure of skeletal size and prior growth because, at this age, wings grow by ~3–6 mm/day (McCarty, 2001); therefore we would not expect to see treatment differences from just 4 h of heat. Measuring both wing and mass in tandem enabled us to distinguish between differences in growth and flexible changes in mass.

2.5 | Quantitative PCR

We quantified relative gene expression using RNA extracted from blood (72 heat and 62 control nestlings), pectoral muscle (25 heat and 20 control nestlings) and hippocampus (25 heat and 17 control nestlings). Due to insufficient RNA, some sample sizes were lower than the number of nestlings sampled. We extracted RNA using Trizol and converted RNA to cDNA using Superscript III (details in Appendix S1C). cDNA was run in triplicate in quantitative real-time PCR (qPCR) to measure mRNA abundance of HSP90AA1. We focused on HSP90AA1 specifically because it is known to elevate within 4 h of heat in birds (Finger et al., 2018) and because HSP90AA1 gene expression was higher in warmer climates in adult tree swallow brains (Woodruff et al., 2022); we later learned HSP90AA1 is the most affected HSP in blood (see below). We calculated mRNA abundance with the comparative Ct method ($2^{-\Delta ct}$): fold change in expression for the gene of interest normalized to an internal reference gene, MRPS25. MRPS25 was stably expressed across treatment groups (<1 Ct difference, on average). Details on qPCR reactions, thermal profiles and primers are in Appendix S1C and Table S2. Plates were balanced by treatment and date. Each plate included intra- and inter-plate controls (a cDNA pool derived from tree swallow RNA). Inter-plate coefficient of variation (CV) was 2.27%, intra-plate CV was 0.55 ± 0.17%.

We measured gene expression in the pectoral muscle because it is the primary flight muscle and the hippocampus because it is a brain area that mediates spatial cognition and stress responses (Madison et al., 2024; Smulders, 2017). Together these traits should affect success after fledging. However, neither of these tissues showed a significant treatment effect on HSP90AA1 gene expression, so we do not discuss them further; see Appendix S1C. We also found no significant effect of sex among the terminally collected samples (Table S4), so we did not pursue this further.

2.6 | RNA-seq and differential gene expression

To shed light on heat-sensitive biological processes beyond HSP90AA1, we submitted total RNA from a subset of blood samples

for RNA-sequencing. This subset included 3 nestlings per treatment (one per nest), balanced by date. We constructed Illumina TruSeq stranded mRNA libraries, and 75-cycle paired-end reads were obtained using an Illumina NextSeq 500. After cleaning, mapping and filtering as in (Bentz et al., 2019), we entered 10,395 genes into a differential expression analysis using DESeq2 (version 1.36.0) in R/Bioconductor (R version 4.2.0; Love et al., 2014); elaborated in Appendix S1D. We functionally analysed differentially expressed genes (DEG) using Gene Ontology (GO) in PANTHER (Mi et al., 2019); we used human reference terms because they are orthologous to, and more complete than, avian references.

2.7 | Fledging and recruitment

To measure fledging success, we checked nests around D21 and identified remaining (dead) nestlings based on their numbered leg band. All 'missing' nestlings were assumed fledged (McCarty, 2001) because all boxes have predator guards and because dead nestlings older than D12 are nearly adult mass, so they are not readily removed by parents (Winkler et al., 2020). We also measured recruitment into the breeding population, devoting substantial effort from March to July the following 2 years to capture and identify breeding birds, including returning nestlings from the experiment. This approach provides a robust estimate of recruitment (Lombardo et al., 2020) because our extensive study population spans 36.4 km—well beyond typical natal dispersal distances for this species (8.38 km for females and 2.44 km for males, Winkler et al., 2005); elaborated in Appendix S1E.

2.8 | Statistical analysis

We used RStudio (2022.07.1 build 554) and JMP (Pro 16.0.0) to conduct three types of analyses: (1) treatment effects on nest temperature, (2) treatment effects on nestling phenotypes and performance and (3) predictors of variation in HSP gene expression, within and among nests. Data and model residuals were inspected for normality via histograms and Q–Q plots. Unless otherwise stated, models assume gaussian distribution. We ensured that model variables were not multicollinear (variable inflation factors <3, Fox & Weisberg, 2018). We report the variance explained by fixed (R^2 marginal) and both fixed and random effects (R^2 conditional) where applicable. We also report effect size (beta estimate, β, or eta squared, η^2, depending on model type) and standard error (SE) for each fixed effect.

2.8.1 | Effects on temperature

To predict mean nest temperature during the experiment, we fit a linear model (LM) with fixed effects of treatment, mean ambient temperature, and brood size. Brood size was included because more nestlings in a confined space may increase heat (Webb & King, 1983).

2.8.2 | Effects on nestling traits

Every model included treatment and mean ambient temperature. We expected the number of nestlings to affect phenotypes because, for example, morphology is related to brood size and behavioural counts are related to the number of nestlings visible on the video. Thus, for behaviour models, we included the mean number of nestlings visible, and for other traits, we included brood size. For models with multiple samples per nest, we included a random effect of nest ID.

To test for heat effects on panting, we ran a negative binomial regression on the count of time intervals *without* any panting; this inversed interval data achieved a better model fit. To test for heat effects on movement toward cooler microclimates, we ran a zero-inflated negative binomial regression on the count of time intervals with at least one nestling positioned head-out-box-hole. Both thermoregulatory behaviour models used the *glmmTMB* package (Brooks et al., 2023). To ease interpretation of figures, we converted the number of 5-s intervals into minutes. To test for heat effects on the proportion of nestling begging, we ran a log-linked binomial regression, a robust approach for proportion data (Chen et al., 2017). To test heat effects on morphology, we ran separate linear mixed effects models (LMM) with dependent variables body mass and wing length. To test for heat effects on HSP gene expression in the blood, we ran a LMM in which mRNA abundance values were Log_2 transformed to improve normality and model fit. We weighted LMMs by treatment group when treatments had unequal variance.

2.8.3 | Effects on fledging and recruitment

These tests used log-linked binomial regressions with a random effect of nest. The likelihood to fledge analysis included 155 nestlings that were not terminally collected. Because all but three nestlings fledged, our model solely tested the main effect of treatment. The likelihood to recruit analysis included 152 fledglings, 13 of which later recruited as adults. For the recruitment analysis, we tested fixed effects of treatment, mean nest temperature, and the interaction between the two because some heat could have positive effects (Dawson et al., 2005; Woodruff et al., 2023) but too much heat could be deleterious (Andreasson et al., 2018; Corregidor-Castro & Jones, 2021; Rodriguez & Barba, 2016; Xie et al., 2018).

2.8.4 | Predicting variation in HSP gene expression

We conducted three types of analyses on variation in HSP gene expression. First, we quantified the scope of within- and among-nest variation in blood HSP gene expression, using coefficients of variation (CVs). Second, to assess whether heat amplified variation in HSP gene expression, we performed a Levene's test on the residuals of our main LMM, thereby contrasting variance between treatments while controlling for confounding effects. To contextualize

this result, we conducted comparable Levene's tests for body mass and wing length, the two other traits we measured at the individual level. Third, we evaluated potential predictors of heat-induced HSP variation, log2-transformed to meet model assumptions. Using individualized data for all heat-exposed nestlings, we tested for an effect of nest ID using a simple ANOVA. We tested whether D12 mass predicted HSP gene expression, using a LMM with a random effect of nest identity. We also explored fixed effects of nest temperature, brood size, amount of panting, and amount of head-out-box hole in separate LMs; because these were measured at the nest-level, our dependent variable was nest-averaged HSP gene expression.

3 | RESULTS

Heat-treated nests were significantly hotter than controls (Figure 1, Tables 1–2, average elevation of 4.5°C). Nest temperatures were higher on hotter days (Figure 1) but were unrelated to brood size (Table 2); model: R^2m = 0.72.

Amount of panting was significantly higher in heated nests (Figure 2a) and on warmer days, but was unrelated to the number of nestlings visible (Table 2); model: R^2m = 0.56. Amount of head-out-box-hole was also significantly higher in heated nests (Figure 2b), on warmer days, and when more nestlings were visible (Table 2); model: R^2m = 0.50. There was no treatment effect on begging intensity (β = −0.30, SE = 0.24, $F_{1,33}$ = 0.03, p = 0.86; Figure S4A), the proportion of nestlings begging (β = 0.39, SE = 1.65, z = 0.24, p = 0.81; Figure S4B), or amount of parental provisioning (β = 0.38, SE = 5.10, $F_{1,33}$ = 0.28, p = 0.60; Figure S5); details in Appendix S1B.

Body mass was lower in heated nests (Figure 2c), and in larger broods, but was unrelated to ambient temperature (Table 2); model: R^2m = 0.23, R^2c = 0.54. On average, heat-exposed nestlings were 19.6 ± 0.2 g and control nestlings were 20.8 ± 0.2 g, a difference of 1.2 g or 5.8%. Wing length was unrelated to treatment (Figure S6), ambient temperature, or brood size (Table 2); R^2m = 0.05, R^2c = 0.45. Treatments did not differ in their variance in wing length (Levene's Test: $F_{1,201}$ = 1.67, p = 0.20) or body mass (Levene's Test: $F_{1,201}$ = 1.77, p = 0.18).

Blood HSP gene expression was higher in heated nests (Figure 3, inset) but was unrelated to ambient temperature or brood size (Table 2); model: R^2m = 0.29, R^2c = 0.71. Blood HSP gene expression showed a significant signature of nest identity (R^2m = 0.58, η^2 = 0.67, SE = 1.06, $F_{23,47}$ = 4.14, p < 0.001), meaning siblings in the same nest were more similar to each other than to the rest of the population. Among heated nests, mean HSP gene expression was unrelated to: nest temperature, brood size, panting, or head-out-box-hole, and at the individual level, heat-induced HSP gene expression was

TABLE 2 Beta estimate effect sizes (β), standard error (SE), test statistic (F or z, depending on the model), and p-value are reported. p < 0.05 are bolded.

Variable	Predictor	β	SE	Test statistic	p value
Nest temp	Treatment	4.05	0.49	$F_{1,42}$ = 86.18	**<0.001**
	Ambient temperature	0.32	0.06	$F_{1,42}$ = 29.04	**<0.001**
	Brood size	0.36	0.24	$F_{1,42}$ = 2.32	0.14
Intervals without panting	Treatment	−0.63	0.25	z = −2.54	**0.01**
	Ambient temperature	−0.14	0.02	z = −5.65	**<0.001**
	# of nestlings visible	−0.04	0.13	z = −0.33	0.74
Intervals with head-out-box-hole	Treatment	1.32	0.60	z = 2.21	**0.03**
	Ambient temperature	0.11	0.05	z = 2.10	**0.04**
	# of nestlings visible	0.63	0.21	z = 3.04	**0.002**
D12 mass	Treatment	−1.02	0.42	$F_{1,41.71}$ = 5.87	**0.02**
	Ambient temperature	0.08	0.10	$F_{1,41.30}$ = 2.65	0.11
	Brood size	−0.80	0.21	$F_{1,42.69}$ = 15.13	**<0.001**
D12 wing length	Treatment	−1.37	1.03	$F_{1,40.70}$ = 1.78	0.19
	Ambient temperature	0.02	0.13	$F_{1,40.31}$ = 0.03	0.87
	Brood size	−0.80	0.50	$F_{1,41.62}$ = 2.51	0.12
BL HSP gene expression	Treatment	1.31	0.30	$F_{1,42}$ = 20.91	**<0.001**
	Ambient temperature	0.05	0.04	$F_{1,42}$ = 1.96	0.17
	Brood size	−0.02	0.15	$F_{1,42}$ = 0.03	0.87
Fledging	Treatment	−0.51	1.24	z = −0.41	0.68
Recruitment	Treatment	47.08	19.75	z = 2.38	**0.02**
	Mean nest temperature	0.31	0.38	z = 0.81	0.42
	Treatment × mean nest temperature	−1.17	0.51	z = −2.29	**0.02**

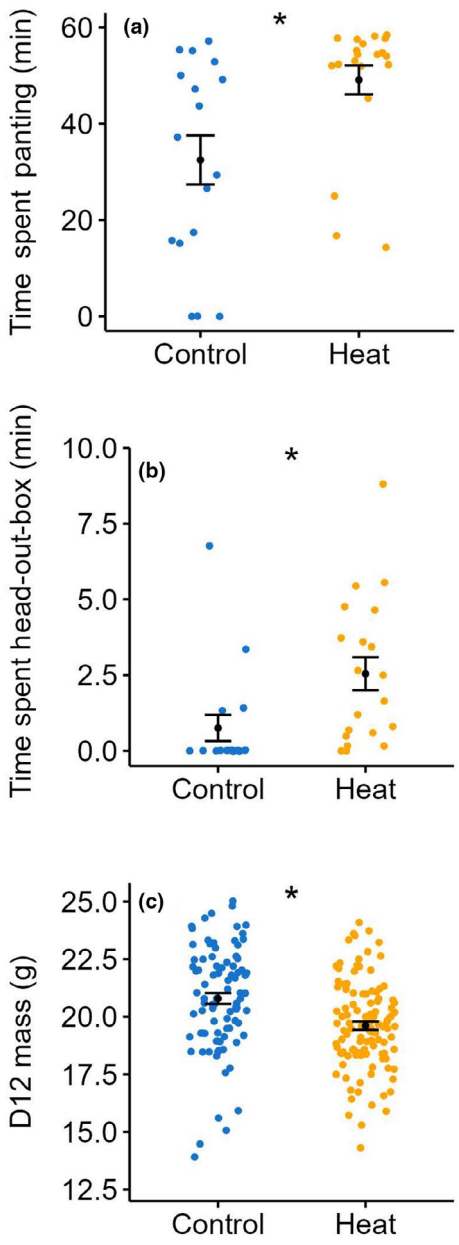

FIGURE 2 Phenotypic effects of heat. Total duration (minutes) at least one nestling was (a) panting or (b) head-out-box-hole during 60 min of observation. Each point represents one nest. (c) Nestling mass (grams) at the end of the experiment. Each point represents one nestling. Mass model accounts for the random effect of nest ID. Behaviour: Heat treatment nest $n = 20$, control nests $n = 17$; D12 Mass: Heat treatment nestlings $n = 112$, control nestlings $n = 91$. Black points indicate treatment group means and error bars are mean ± SE. Asterisk indicates difference between treatments ($p < 0.05$).

unrelated to body mass ($p \geq 0.16$); Table S5 and Figure S8. For the heat treatment, within-nest CV was 59.3% (range = 11.6%–130.1%) and among-nest CV was 159.4%. For the control treatment, mean within-nest CV was 43.0% (range = 8.0%–84.1%) and among-nests CV was 79.1%. Variance in blood HSP gene expression differed between treatments (Levene's Test: $F_{1,132} = 5.88$, $p = 0.02$).

We identified 92 DEGs in the blood (Table S6; see Table S7 for all 794 DEG prior to false discovery rate correction). HSP90AA1 was the second most affected among these DEG. GO analyses identified several enriched biological processes, including *protein folding*, *myeloid cell differentiation*, *response to heat*, *response to hormone*, and *negative regulation of apoptotic process* (full list in Table S8). Genes within these terms were largely upregulated and included several additional heat shock proteins (e.g. DNAJA1, DNAJB4, HSP90AB1, HSPA2, HSPA4L), plus others related to antioxidants (PRDX4, BIEA, HMOX1, GSTZ1), inflammation (IL1B, TLR2, IFNAR1), metabolism (IRS4, PDK2), and ubiquitination (UBC, MAEA).

Four hours of heat had no effect on the likelihood to fledge (Table 2; all fledged except 1 control and 2 heat; R^2m = 0.02, R^2c = 0.02). However, recruitment was significantly predicted by the interaction between treatment and nest temperature (Table 2; R^2m = 0.30, R^2c = 0.30) with higher recruitment among the coolest of the heat-exposed nests (Figure S9). Of the 13 birds that recruited, 10 were from heated nests and 3 were from control nests. Recruitment rates of 4% in controls and 12% in heat are low but typical for this migratory species (Winkler et al., 2020).

4 | DISCUSSION

We temporarily elevated nest temperatures by 4.5°C, to an average of 40.9°C, simulating an afternoon we might expect with climate change (Reidmiller et al., 2018). In experimental nests, we documented higher rates of thermoregulatory behaviours but no effect on nestling begging or parental provisioning. Nestling mass was lower in the heated group despite no treatment differences in wing length, consistent with evaporative water loss via panting. Four hours of heat also induced high levels of blood HSP gene expression, alongside other transcriptional changes related to antioxidant defences, inflammation, and apoptosis. Heat had no effect on the likelihood of fledging, supporting the sub-lethal nature of the challenge; however, nestlings exposed to milder heat were more likely to be recaptured as adults, suggesting a positive carryover effect of short-term early life experience with some degree of heat. These results unveil the mechanisms that compose a nestling's response to a short-term but naturalistic heat challenge.

Behavioural thermoregulation is thought to be a first line of defence against heat (reviewed by: Huey et al., 2012; Muñoz, 2022), but sessile organisms (Pandolfi et al., 2011) and altricial young (Larson et al., 2015) may have limited options. Here, heat-exposed nestlings spent more time at the nest box entrance. Enclosed nests, including nest boxes, are naturally hotter than ambient, so this movement toward the box hole may enable access to cooler air. Notably, these benefits may not be accessible to all nestmates because the box hole is about the width of one nestling. Indeed, across all observations, this behaviour was largely limited to just one nestling (~50% of cases) or two nestlings (~30% of cases), suggesting that some individuals may dominate access to thermal refuges (Cunningham et al., 2017). If the benefits of behavioural thermoregulation are

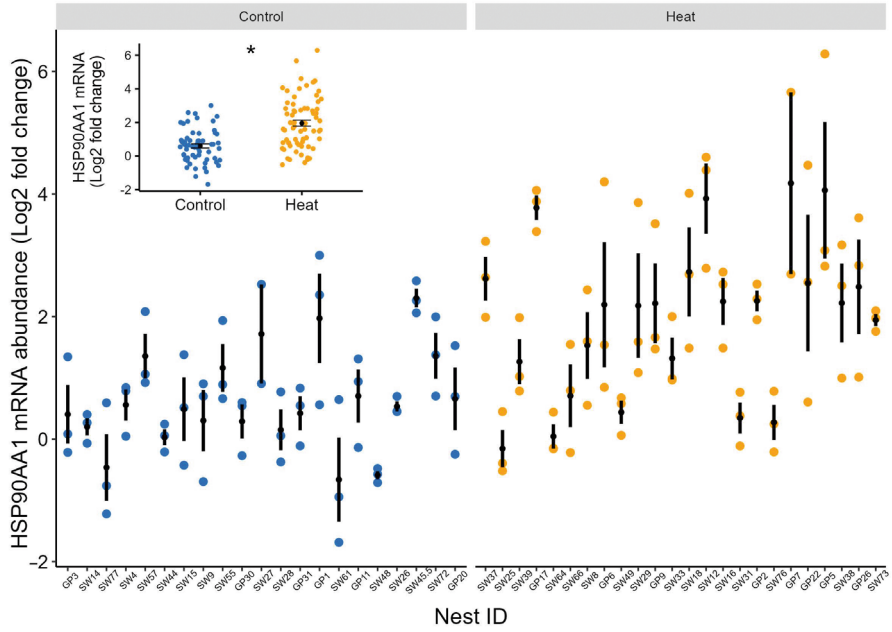

FIGURE 3 Relative gene expression of blood HSP90AA1 ($Log_2\ 2^{-\Delta ct}$) difference between treatments (inset) and among nests. Within each treatment, nests are ordered by increasing mean nest temperature. Each point represents one nestling. Nestlings per nest $n = 2–3$. Nestmate plot: Heat treatment nests $n = 24$; control nests $n = 21$. Inset plot: Heat treatment nestlings $n = 72$, control nestlings $n = 62$. Black points indicate nest means and error bars are mean ± SE. Asterisk indicates difference between treatments ($p < 0.05$). Note that 1 unit is a 2-fold difference in abundance on this log2-scale.

density-dependent in confined burrows or nests, brood or litter sizes may be constrained, contributing to declines in global bird populations (Halupka et al., 2023).

Panting is another common heat coping behaviour (Loughran & Wolf, 2020), but dehydration from evaporative water loss represents a real concern. We observed 1.2 g, or 5.8%, lower mass after 4 h of heat. We attribute this result to panting-induced water loss because treatments did not differ in structural size (wing length) or apparent food intake (parental provisioning). This also means that risk of heat-induced dehydration extends beyond passerines in arid climates (Albright et al., 2017) into temperate, wet climates like that of the tree swallow. With forecasted warming as much as 5°C this century (Reidmiller et al., 2018), nestling birds may be vulnerable, especially when temperatures last more than a few hours.

As we seek to understand how altricial animals mitigate heat when their behavioural repertoire is still limited, acute physiological responses may be critical. Our RNA-seq analyses highlight several such pathways, including upregulation of HSPs that serve to limit heat-induced protein damage (Feder & Hofmann, 1999; Lindquist & Craig, 1988). We also documented increased transcription along pathways of antioxidation (e.g. PRDX4, BIEA, HMOX1, GSTZ1), pro-inflammatory signalling (e.g. IL1B, TLR2, IFNAR1), and ubiquitination, the latter of which marks damaged elements for destruction (e.g. UBC, MAEA). To the degree that these patterns translate to functional heat mitigation, these data represent a key step toward the development of transcriptional assays that can be applied across species that vary in their sensitivity to heat (Taff & Shipley, 2023).

HSP90AA1 was the HSP with the strongest transcriptional response, which was also highly variable, especially after heat exposure. In terms of fold-differences in gene expression, nestmates differed from one another by an average of 2.6-fold in controls (range: 1.2 to 5.4-fold), with up to 26-fold differences across the population. After heat exposure, though, nestmates differed from one another by an average of 4.3-fold (range: 1.3 to 14.6-fold), and heat-exposed individuals in the population differed by as much as 112-fold. This high variance after heat exposure is consistent with the idea that stressful or novel environments may reveal cryptic phenotypic variation (Ghalambor et al., 2007; Jarosz et al., 2010). That HSP variation was enhanced by heat, whereas variance in other traits was not, underscores previous laboratory research suggesting that HSPs may potentiate phenotypic change (Jarosz & Lindquist, 2010; Rutherford & Lindquist, 1998; Tanner et al., 2022). We extend these ideas, showing they apply to wildlife responding to real-time heat. As hot afternoons become more frequent, then this variation may become more visible to selection, and populations with a high degree of standing variation should fare better (Hoffmann & Sgrò, 2011).

Why do nestlings vary so much in blood HSP gene expression? Our analyses shed light on some possible sources of variation, while highlighting key questions for the future. For example, nest identity explained 58% of the variation in HSP gene expression. This result provides a maximum value for heritability, acknowledging that nestmates share the same developmental environment and they may be half-siblings (Whittinghan et al., 2006). We were surprised that HSP gene expression was unrelated to body mass, considering larger individuals may have lower heat tolerance (Gunderson et al., 2019). However, size-dependent effects on physiology could be masked by the thermal inertia of larger bodies (Gunderson, 2024) or if size determines access to cooler microclimates (Gunderson et al., 2019), like the cavity entrance. Brood size and subtle variation in temperature among heated nests did not predict differences in HSP mRNA abundance either. We also note that heat effects on HSP gene expression were unique to the blood, though we cannot disentangle the mechanism of this result here (details in Appendix S1C). With several biotic and abiotic factors accounted for, behavioural differences among individuals remain unexplored, particularly since we quantified behaviour at the nest level and did not capture any among-individual

behavioural coping that may shape the 'need' for a physiological response (Lipshutz et al., 2022).

As an added layer of complexity, the tree swallow breeding range has been expanding *south* in the last few decades, into the warm and humid southeastern United States (Shutler et al., 2012; Wright et al., 2019), counter to most species that are shifting to higher latitudes or altitudes (Chen et al., 2011). The ecological drivers of this expansion are still unclear (Shutler et al., 2012; Siefferman et al., 2023), but the pattern suggests some degree of coping well with heat. Our experiment bolsters this view, with a single warm afternoon positively affecting nestlings' likelihood to recruit in following years, and recruits more likely from the coolest heated nests. Whether such carryover effects stem from elements of environmental matching, condition dependent survival, or something else is not yet clear (reviewed by Nord & Giroud, 2020), but our results show that—up to some limit—heat-exposed nestlings fared well in the longer term.

Rising temperatures have the potential to drive species trajectories (McKechnie & Wolf, 2019), and the scope of individual variation should shape adaptive potential (Hoffmann & Sgrò, 2011). Our results shed light on oft-ignored elements of thermotolerance in wild birds at a critical stage of post-natal development. Further, we highlight the scope of heat-induced HSP gene expression and couple it with a suite of organismal traits. Together, our organismal perspective provides a generalizable framework for testing the valence of acute heat responses. This multi-trait approach provides a holistic understanding of how thermotolerance is built during this time of unprecedented change.

AUTHOR CONTRIBUTIONS

Conceptualization: Mary J. Woodruff and Kimberly A. Rosvall. Investigation: Mary J. Woodruff, Susanna N. Tsueda, Tiernan S. Cutrell, Ethan A. Guardado, Douglas B. Rusch and Aaron Buechlein. Data curation: Mary J. Woodruff, Susanna N. Tsueda, Ethan A. Guardado, Douglas B. Rusch and Aaron Buechlein. Formal Analysis: Mary J. Woodruff, Susanna N. Tsueda, Ethan A. Guardado, Douglas B. Rusch and Aaron Buechlein. Project administration: Mary J. Woodruff. Supervision: Kimberly A. Rosvall. Writing—original draft Mary J. Woodruff and Kimberly A. Rosvall with feedback from all authors.

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CONFLICT OF INTEREST STATEMENT

All authors have no competing interests.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.dncjsxm8f (Woodruff et al., 2024).

STATEMENT OF INCLUSION

All authors lived locally to the study area, including three undergraduates from the region. All authors were authentically involved throughout the project.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix 1. Supplemental Methods and Information.

Figure S1. Experimental set-up.

Figure S2. Nest temperatures (°C) measured before and during the experiment, for heat-exposed nests only.

Figure S3. Nest versus ambient percent relative humidity.

Figure S4. Treatments did not differ in (A) mean begging intensity and (B) mean proportion of nestlings begging per feed during the 1 hr observation period.

Figure S5. Treatments did not differ in the total number of parental feeds during the 1 h. observation period.

Figure S6. D12 nestling wing length (mm) at the end of the experiment did not differ by treatment.

Figure S7. Treatments did not differ in HSP90AA1 gene expression, in the (A) hippocampus or (B) pectoral muscle.

Figure S8. Heat treatment group blood HSP90AA1 relative gene expression by (A) mean nest temperature, (B) brood size, (C) time spent panting, (D), time spent head-out-box-hole and (E) D12 body mass.

Figure S9. Relationship nest temperature during the experiment and later recruitment.

Table S1. Results of inter-rater reliability.

Table S2. Primer sequences, efficiencies and citations.

Table S3. Relative humidity (% RH). Mean nest values come from 10 min iButton values, averaged per box across the duration of the experiment (± standard error).

Table S4. Linear model results testing for sex differences in log2 HSP90AA1 gene expression.

Table S5. Variance explained by fixed effect (R^2m), variance explained by both fixed and random effects (R^2c) for applicable model, beta estimate effect sizes (β), standard error (SE), test statistic and *p*-value are reported.

Table S6. Significantly differentially expressed genes in nestling blood after FDR.

Table S7. Significantly differentially expressed genes in nestling blood prior to FDR.

Table S8. GO terms for differentially expressed genes in nestling blood relative to controls.

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