



How thermal challenges change gene regulation in the songbird brain and gonad: Implications for sexual selection in our changing world

Sara E. Lipshutz¹  | Clara R. Howell² | Aaron M. Buechlein³ | Douglas B. Rusch³ | Kimberly A. Rosvall¹  | Elizabeth P. Derryberry²

¹Department of Biology, Indiana University, Bloomington, Indiana, USA

²Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, Tennessee, USA

³Center for Genomics and Bioinformatics, Indiana University, Bloomington, Indiana, USA

Correspondence

Elizabeth P. Derryberry, Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN, USA.

Email: liz@utk.edu

Present address

Department of Biology, Loyola University Chicago, Chicago, IL, USA

Department of Biology, Duke University, Durham, NC, USA

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Abstract

In a rapidly warming world, exposure to high temperatures may impact fitness, but the gene regulatory mechanisms that link sublethal heat to sexually selected traits are not well understood, particularly in endothermic animals. Our experiment used zebra finches (*Taeniopygia guttata*), songbirds that experience extreme temperature fluctuations in their native Australia. We exposed captive males to an acute thermal challenge (43°C) compared with thermoneutral (35°C) and lower (27°C) temperatures. We found significantly more heat dissipation behaviours at 43°C, a temperature previously shown to reduce song production and fertility, and more heat retention behaviours at 27°C. Next, we characterized transcriptomic responses in tissues important for mating effort—the posterior telencephalon, for its role in song production, and the testis, for its role in fertility and hormone production. Differential expression of hundreds of genes in the testes, but few in the brain, suggests the brain is less responsive to extreme temperatures. Nevertheless, gene network analyses revealed that expression related to dopaminergic signalling in the brain covaried with heat dissipation behaviours, providing a mechanism by which temporary thermal challenges may alter motivational circuits for song production. In both brain and testis, we observed correlations between thermally sensitive gene networks and individual differences in thermoregulatory behaviour. Although we cannot directly relate these gene regulatory changes to mating success, our results suggest that individual variation in response to thermal challenges could impact sexually selected traits in a warming world.

KEYWORDS

environmental change, thermal stress, transcriptomics, zebra finch

1 | INTRODUCTION

Life on Earth is currently experiencing the highest temperatures in recorded history (Wallace-Wells, 2020). Whereas extreme heat anomalies were limited to <1% of the Earth's surface in the past,

extreme events now cover more than 10% of the land area (Hansen et al., 2012). Despite this global challenge, we know relatively little about the impact of heat waves on terrestrial animal populations (Stillman, 2019). Furthermore, the effects of heat on behaviour and physiology are far less well understood in endotherms than

in ectotherms (Angilletta, 2009; Buckley & Kingsolver, 2021; Soravia et al., 2021; Wingfield et al., 2017). Yet, the potential for heat waves to cause death in endotherms is well documented, with mass mortality events in birds (McKechnie & Wolf, 2010; Riddell et al., 2021), small mammals (Welbergen et al., 2008) and humans (Zhao et al., 2018). There is growing evidence that high temperatures also affect fitness-related behaviours in endotherms, such as mating (Coomes et al., 2019; Coomes & Derryberry, 2021) and foraging (Danner et al., 2021), and that these sublethal effects occur at temperatures well below those that cause death (Walsh et al., 2019). Thus, sublethal, behavioural effects of heat may be essential to predicting species' persistence in the face of rising temperatures (Conradie et al., 2019; Soravia et al., 2021), underscoring the urgent need to identify the scope of these effects and how they arise.

Among those behaviours affected by heat, mating effort is of particular interest, because obtaining a mate is a key determinant of fitness. Songbirds are particularly vulnerable to heat waves because they are diurnal and make limited use of microclimates for cooling (McKechnie & Wolf, 2010); even when inactive in complete shade, small species can rapidly dehydrate (Wolf & Walsberg, 1996). Wild male songbirds also sing less in hot weather (Luther & Danner, 2016), as do domesticated songbirds when exposed to an acute thermal challenge (Coomes & Derryberry, 2021). Thermal challenges can alter the signal itself, with song duration shortening as ambient (Coomes & Derryberry, 2021) and brain (Long & Fee, 2008) temperatures increase. As female songbirds often prefer males with greater song output and longer songs (Catchpole & Slater, 2008), high temperatures may act as a selective pressure impacting fitness through reduced mate attraction, particularly if males vary in their capacity to mitigate the effects of heat. Heat may also have direct effects on fertility in the testis, the site of sperm and reproductive hormone production (Hansen, 2009; Wang, Cheng, et al., 2015; Wechalekar et al., 2010). Even in species with internal testes, like birds, exposure to elevated temperatures can reduce sperm or semen quality (Hurley et al., 2018; Murugesan et al., 2017). Altogether, these studies suggest there are multiple avenues through which heat may affect mating effort or the outcome of sexual selection.

Molecular responses at the tissue level may shed light on exactly how thermal challenges affect mating effort. One avenue through which heat could negatively impact mating effort is by causing animals to increase heat dissipation behaviours, such as panting, at the expense of investment in mating behaviours (Campagna & Le Boeuf, 1988; Luther & Danner, 2016; Mitchell et al., 2018). In this case, we might expect that heat dissipation behaviours will vary with gene regulatory networks enriched for cellular *self-maintenance* at high temperatures, for example by upregulating molecular chaperones like heat shock proteins (Chen et al., 2018), which combat and repair damage (Feder & Hofmann, 1999). Another avenue is that heat may alter *behavioural circuits in the brain* (Angilletta et al., 2018; Rummel et al., 2018) via effects on neuropeptides or hormones (Sun et al., 2015; Tu et al., 2016). For

example, motivation to sing may be reduced if heat affects dopaminergic signalling (Kim et al., 2013) or, alternatively, birds may be equally motivated to sing under thermal challenges, but unable to do so because of impaired motor function or trade-offs with the increased demands of self-maintenance outside the thermoneutral zone. In addition, heat may affect *testicular traits*, like steroidogenesis (Li et al., 2020) or spermatogenesis (Nakai et al., 2000), with potential downstream effects on primary reproductive traits (Wang, Cheng, et al., 2015) or secondary sexual traits, like mate attraction behaviours. Notably, these are not mutually exclusive hypotheses because heat dissipation behaviours have the potential to mitigate the effects of heat via avenues of increased self-maintenance, altered neural mechanisms and altered gonadal function. Previous research in ectotherms suggests that there is marked variation in thermal sensitivity among tissues (Logan & Buckley, 2015), but outside of livestock (e.g., Rout et al., 2016) most comparisons in endotherms are limited to candidate thermally regulated genes. Although there are many mechanisms that animals use to mitigate the effects of heat, genome-wide expression is a powerful approach to understand transcriptomic responses to thermal challenges because it connects rapid changes in the environment to activation of the genome (Rivera et al., 2021; Watson et al., 2017).

Here, we administered acute thermal challenges to evaluate these alternative hypotheses by which heat affects mating effort. We quantified functional enrichment of heat-sensitive genes and networks, and we measured correlations between thermoregulatory behaviour and gene activity in two tissues with close ties to mating effort. We focused on neural and gonadal tissues from domesticated male zebra finches at a high temperature ($T_a = 43^\circ\text{C}$) as compared to a temperature within their thermoneutral zone ($T_a = 35^\circ\text{C}$, hereafter 'TNZ'; Calder, 1964; Wojciechowski et al., 2020). Exposure to $T_a > 40^\circ\text{C}$ poses a physiological challenge in this species (Cooper et al., 2019; Cooper, Hurley, & Griffith, 2020; Wojciechowski et al., 2020). Additionally, we added a second thermal comparison ($T_a = 27^\circ\text{C}$) to distinguish between heat-specific responses and more general responses to temperature change outside of the TNZ. In zebra finches, 27°C is below thermoneutrality (Calder, 1964; Wojciechowski et al., 2020). We predicted that individuals would respond to air temperatures outside of their TNZ by increasing their thermoregulatory behaviours, such as panting to keep cool (heat dissipation) and piloerection to keep warm (heat retention). We predicted that individual variation in these thermoregulatory behaviours would correlate with gene regulatory responses involved in minimizing thermal damage at high temperatures (e.g., protein folding, repair of cell damage, apoptosis, inflammation) as well as those involved in regulating mating effort in the brain or gonad (e.g., sex steroid hormones, neurotransmitters). We predicted that the scope of outcomes would vary between tissues, assessed as the number of differentially expressed genes and relative alteration of gene regulatory networks. Testing these hypotheses will provide insight into the potential molecular mechanisms by which behavioural responses to rapid environmental change can impact mating effort.

2 | MATERIALS AND METHODS

2.1 | Study system

As an arid-zone bird, the zebra finch (*Taeniopygia guttata*) is a sentinel species for behavioural and physiological responses to the ever-increasing threat of heat. Like most songbirds, zebra finches are vulnerable to heat waves, and can suffer mass mortality events, with mortality expected above 45°C (Finlayson, 1932; McKechnie et al., 2012). Zebra finches also experience extensive periods of sublethal temperatures (Danner et al., 2021) and so assessing the effects of heat on behaviour is biologically relevant for this species (Funghi et al., 2019).

Zebra finch body temperature is typical for passerines (~42°C) and their thermal neutral zone is ~29.5–40°C (Cade et al., 1965; Calder, 1964) although recent work suggests it may be much narrower (34.87–37.47°C) (Wojciechowski et al., 2020). During breeding periods, zebra finches experience average ambient temperatures ranging from 2.2 to 36°C (Zann, 1996), and maximum temperatures regularly exceed 40°C (Griffith et al., 2016). Although they can show physiological flexibility during heatwaves in the wild (Cooper, Hurley, Deviche, & Griffith, 2020), zebra finches are subject to multiple fitness consequences from extreme heat events. Namely, at high temperatures close to or above the upper critical temperature of TNZ, zebra finches experience a reduction in sperm quality (Hurley et al., 2018), forage less often and are less social (Funghi et al., 2019), change their egg morphology (Hoffman et al., 2021), and sing more to their embryos, which changes how nestlings grow and behave (Katsis et al., 2021; Mariette & Buchanan, 2016; Pessato et al., 2020). Importantly, domesticated zebra finches do not differ from wild birds in their thermal physiology (Calder, 1964; Marschall & Prinzinger, 1991), making them an excellent model for understanding responses to thermal challenges.

2.2 | Subjects and housing conditions

We obtained 24 adult male zebra finches from Magnolia Farms Avian Breeder. Prior to purchase, birds were housed in heated outdoor aviaries that experience typical maximum temperatures of 27.7°C in the summer and typical minimum temperatures of 8.3°C (mitigated by heat lamps) in the winter (based on climatological data for Orange County, CA, USA; NOAA). After purchase, birds were housed in an indoor vivarium with an average daily temperature of 24–25°C, with a high of 26°C and a low of 23°C.

Birds were housed in a group cage for 4 days after arrival, after which they were rehoused in individual cages (48×25×30 cm) for 2 weeks before experimentation. Food and water were provided ad libitum. The room was illuminated on a 13:11-h light–dark cycle with full spectrum lights, which encourages reproductive readiness in this opportunistically breeding bird (Beaulieu, 2016; Olson et al., 2014). All methods were approved under IACUC Protocol 2578.

2.3 | Temperature manipulations

Temperature manipulations occurred in six sound attenuation chambers (Industrial Acoustic; MAC 1) located in the vivarium that were modified for accurate temperature control ($\pm 0.3^\circ\text{C}$) with consistent uniformity ($\pm 1^\circ\text{C}$) across a broad range of temperatures (22–44°C). All chambers had vents and fans that allowed for continual circulation of air, temperature probes to monitor chamber temperature and two cameras. Chambers were assigned to each treatment group on a rotating block schedule such that each chamber was assigned to each temperature treatment at least once.

The experiment began by sliding the cage into the chamber (i.e., subjects were not handled), followed by 36 h of acclimation to the chamber, and subsequent exposure to one of three temperatures: 27°C ('cold'), 35°C ('TNZ') or 43°C ('hot'). For logistical purposes, start times were staggered, beginning at least 1 h after lights on and ending 5.2 ± 0.06 h (mean \pm SE) later. Subtle variation in timing stems from limited variability in temperature ramp time among chambers. We chose this 5-h treatment duration because this length of heat exposure is sufficient to change thermoregulatory behaviour in songbirds (Danner et al., 2021) as well as gene expression in both brain (Tu et al., 2016) and testes (Wang, Cheng, et al., 2015) in poultry. Furthermore, this length of exposure is ecologically relevant. During a well-documented 8-day heat wave in Fowler's Gap, Australia, in February 2017, free-living zebra finches experienced temperatures exceeding 43°C for ~5 h a day on three of those days (Hurley et al., 2018).

2.4 | Behavioural analysis

Logitech webcams were placed in the upper-right and upper-left corners of each chamber, allowing for almost complete coverage of the chamber. Videos were cut and synced using IMOVIE. Recording occurred during the full ~5-h manipulation, but our analyses focused on the final 15 min before tissue collection. These excerpts were renamed by an independent observer so that behavioural scoring would be done blindly and in a randomized order. All videos were scored by the same individual (C.H.) using the Animal Behaviour Pro app (Newton-Fisher, 2012).

The following behavioural states were recorded. (i) *Panting* was defined as an open beak and the bird breathing with some rapidity. Birds typically open and close their beak during a panting session. A closed beak indicates a cessation in panting when it has been closed for ~3 s. If the beak is closed for longer than 3 s, it typically stays closed for some time until panting resumes, whereas shorter time intervals frequently lead to resumed panting. (ii) *Piloerection* was defined as erected body feathers for longer than 1 s. Piloerection was typically visible via extended feathers and a larger-looking body size. The duration of these states (in seconds) was used to calculate the percentage time spent in each state during the sampled periods of time.

Although initially treatment groups had eight individuals, camera malfunctions made only six in each group available for analysis. Thus, behavioural analyses were performed on six birds per group. We compared behaviours and bill surface area across treatments using Kruskal–Wallis tests with pairwise Wilcoxon rank sum tests in R (R Core Team, 2021).

2.5 | Tissue collection

At the end of temperature treatment, birds were killed with an overdose of isoflurane, followed by decapitation (average time from chamber removal to death = 48.3 ± 2 s). Using tools cleaned with RNase-away and 95% ethanol, we dissected whole brains and testes, and froze tissues on powdered dry ice (average time from death to brain freezing = $3 \text{ min } 21 \pm 1$ s, average time to testis freezing = $4 \text{ min } 26 \pm 3$ s). Testes were enlarged, indicative of males in breeding condition. For two individuals, one from the cold treatment group and one from the hot treatment group, the testes lost structure during dissection and were excluded from further analyses. Thus, our sample sizes for neural tissue analyses were $n = 8$ per treatment group, and our gonadal tissue sample sizes were $n = 7$ for hot, $n = 7$ for cold and $n = 8$ for TNZ.

2.6 | Morphological measurements

The bill is an important mechanism for heat dissipation in birds, and so we considered how this morphological trait varied with behavioural and gene regulatory responses to heat. To minimize disturbance prior to the experiment, we recorded intact bill measurements post-mortem following dissection. Bill depth and width were measured at the nares. Bill length was measured from the tip to the nares. Bill surface area was calculated by treating the bill as a cone (Greenberg et al., 2012; Luther & Danner, 2016): $\text{bill length} \times \pi \times ((\text{bill width} + \text{bill depth}) \div 4)$. Treatment groups did not differ in bill surface area ($F_{2,20} = 0.56, p = .58$).

2.7 | Tissue dissection and RNA isolation

We macrodissected whole brains into 12 functional regions following Soma et al. (1999, 2003). We focused on the right hemisphere of the posterior telencephalon (hereafter PTR), because it includes the song control nuclei HVC and RA (Jarvis et al., 1998; Margoliash, 1997), and the testis, because of its role in spermatogenesis and testosterone production (Aire, 2007; Ubuka et al., 2014). We extracted total RNA integrity number (RNA) from PTR and testis samples using the phenol–chloroform–based Trizol method (Invitrogen). We resuspended RNA in UltraPure DNase/RNase-free distilled water (Invitrogen) and assessed quantity/quality with a TapeStation 2200 (Agilent). Mean sample RIN was 9.09 ± 0.03 .

2.8 | RNAseq and differential gene expression

We submitted total RNA to Indiana University's Center for Genomics and Bioinformatics for sequencing. Illumina sequencing libraries were generated using the TruSeq Stranded mRNA HT Sample Prep Kit following the standard manufacturer's protocol. Sequencing was performed using an Illumina NextSeq 500 platform with a 75-cycle sequencing module/550 High Output Kit v2 generating 38-bp paired-end reads or 75-bp single-end reads. Brain samples consisted of only paired-end reads while gonad samples included both single- and paired-end reads. Sequencing reads were demultiplexed with BCL2FASTQ. The reads were adapter and quality trimmed using TRIMMOMATIC version 0.36 (Bolger et al., 2014). Trimmed reads were mapped to the reference Zebra finch genome (Taeniopygia_guttata-3.2.4.98) (Warren et al., 2010) assembly using HISAT2 version 2.1.0 (Kim et al., 2013). HISAT uses BOWTIE2, which is based on the Burrows–Wheeler transform algorithm, for sequence alignment and allows for mapping across exon junctions (Langmead & Salzberg, 2012). When applicable, results were filtered to include only reads mapped in proper pairs. Reads were sorted and indexed using SAMTOOLS version 1.9 (Li et al., 2009). For PTR samples, ~49 million read pairs per sample were mapped, which account for ~78.9% of the total read pairs (Table S1). For testis samples, ~27 million inserts per sample were mapped, which account for ~77.5% of total inserts (Table S1). Reads were quantified into genes using featureCounts from the SUBREAD package version 1.5.3 with the parameters “-O -M --primary --largestOverlap -s 2 -B” (Liao et al., 2019). Read quantification was done separately for both single- and paired-end reads, then compared and found to be highly concordant (Pearson's $r > .99$). We identified 16,015 genes in the brain and 17,051 genes in the gonad. In total, 15,819 genes were expressed in both tissues (Figure S1).

Differential gene expression analysis was performed using the DESeq2 package (version 1.24.0 for PTR and v 1.26.0 for testis) in R/BIOCONDUCTOR (R version 3.6.0) (Love et al., 2014). Briefly, the data are fitted to a negative binomial generalized linear model with a fixed effect for temperature treatment for each transcript and filtered based on a per-transcript Wald test statistic to identify valid significantly differentially expressed transcripts. For each tissue, we used \log_2 foldchange to compare means of gene expression between treatment groups: TNZ vs. hot, and TNZ vs. cold. The p -values were corrected for multiple-testing (Benjamini–Hochberg; adjusted $p \leq .05$). Relative rank abundance was determined using the normalized read counts generated by DESeq2. For gonadal samples, we used a model that incorporated paired- and single-end status. Scripts and input files for DESeq2 are available in the Dryad Digital Repository: doi:10.5061/dryad.j9kd51cdf (Lipshutz et al., 2022; <https://doi.org/10.1111/mec.16506>). For each set of differentially expressed genes, we inferred gene ontology (GO) terms using *Homo sapiens* as the reference because its ontologies are orthologous to, but more complete than, avian references. We conducted an overrepresentation analysis of biological process GO terms in WEBGESTALT (Liao et al., 2019).

We also compared variability in differential gene expression between temperature contrasts for each tissue. For each of the four contrasts, we created density plots for the top 500 genes with the

lowest adjusted p -value and compared the standard error of \log_2 fold changes for each gene.

2.9 | Weighted gene co-expression networks and GO enrichment analyses

We used weighted gene co-expression network analyses (WGCNA) to identify modules of coregulated temperature-sensitive genes (Langfelder & Horvath, 2008). We had four primary contrasts: TNZ vs. hot, and TNZ vs. cold, replicated for neural and gonadal tissues. We used these dyadic contrasts, rather than a three-way contrast within each tissue because we had no a priori expectation for hot and cold to engage the same networks. Scripts and input files for WGCNA are available in the Dryad Digital Repository: doi:10.5061/dryad.j9kd51cdf (Lipshutz et al., 2022; <https://doi.org/10.1111/mec.16506>).

Networks were constructed using $n = 16$ for neural tissue contrasts and $n = 15$ for gonadal tissue contrasts. To filter out genes with low expression, we removed genes with <10 normalized counts in 87% (14 out of 16) of samples. To filter out genes with low variance, we used the 75% most variable genes, leaving: 9288 genes for PTR TNZ vs. hot, 9296 genes for PTR TNZ vs. cold, 8879 for testis TNZ vs. hot, and 8859 for testis TNZ vs. cold. We built signed hybrid networks using a biweight midcorrelation (bicor) function and soft threshold power (β) = 10 for PTR contrasts and gonad TNZ vs. cold, and $\beta = 9$ for gonad TNZ vs. hot, in accordance with scale-free topology. Minimum module size was set to 30, and modules with similar expression profiles were merged using Dynamic Tree Cut (threshold = 0.25), since these genes are highly co-expressed.

Next, we correlated module eigengenes (i.e., first principal component of expression levels) with traits of interest, including treatment, thermoregulatory behaviours like panting, and piloerection, as well as a morphological trait, bill surface area. Most of these analyses used the full $n = 16$ (neural) or $n = 15$ (gonadal) sample sizes, but behavioural analyses were reduced to $n = 12$ (neural) and $n = 11$ (gonadal). We identified genes with the highest network connectivity, assessed by module membership (i.e., the correlation between gene expression profile and module eigengene), focusing more closely on genes with module membership ≥ 0.6 . Although we do not know the protein-level consequences of these gene regulatory changes, gene expression does broadly predict translation (Lu et al., 2007; Li et al., 2014; Mayfield et al., 2016). Therefore, we again analysed nonredundant functional enrichment of biological process GO terms via WEBGESTALT (Liao et al., 2019).

3 | RESULTS

3.1 | Treatment effects on thermoregulatory behaviours

The three treatment groups differed significantly in time spent in each of the two behavioural states: panting ($\chi^2 = 14.06$, $df = 2$, $p = .00089$) and piloerection ($\chi^2 = 13.27$, $df = 2$, $p = .0013$) (Figure 1).

Compared to the 35°C treatment group, we found more panting at 43°C ($p = .0072$) and more piloerection at 27°C ($p = .019$), indicating that thermal treatments affected behavioural responses. Birds in the 43°C group spent an average of 75.2% of their time panting, with all individuals panting for at least half of the observation. Birds in the 27°C group spent on average 53.3% of their time piloerecting, although individuals in this group varied extensively in the time spent engaged in this behaviour. In comparison, birds in the 35°C group spent less than 5.1% of their time panting or piloerecting. These behavioural data confirm that the 43 and 27°C temperature treatments initiated typical heat dissipation and heat retention behaviours, respectively, when compared to the thermoneutral (35°C) condition. Notably, beak surface area did not explain variation in time spent panting ($r = -.0003$, $p = .99$) or piloerection ($r = -.22$, $p = .39$) across treatment groups.

3.2 | Treatment effects on differentially expressed genes

We present the top three differentially expressed genes per tissue in Figure S2. Looking first at the posterior telencephalon, we saw seven genes (0.04% of total transcripts) that were differentially expressed between hot vs. TNZ treatments (Figure 1; Table S2). These genes were EFTUD2, ATP2A2, ABHD3, MOCS2, FABP7, PALLD and RBM12. EFTUD2 is a GTPase that regulates splicesosome dynamics (Fabrizio et al., 1997), and ATP2A2 encodes a Ca^{2+} ATPase that is known to regulate cellular maintenance under stress conditions (Cheviron & Swanson, 2017; Wood et al., 2019). No genes were differentially expressed in the brain between cold vs. TNZ treatments (Figure 1; Table S3).

In contrast in the gonad, 925 genes (5.4% of total transcripts) were differentially expressed between hot and TNZ treatments (Figure 1; Figure S1 and Table S4). This set of differentially expressed genes was significantly enriched for cell morphogenesis and cell projection organization (Table S5). Genes differentially expressed between hot and TNZ treatments in the gonad also included heat shock proteins: BAG2, HSPA2, HSPA4, DNAJB5, DNAJB11, DNAJC3 and DNAJC15. Additionally, in the gonad, 268 (1.6% of total transcripts) were differentially expressed between cold and TNZ treatments (Figure 1; Figure S1 and Table S6). One gene encoding a heat shock protein, DNAJB11, was included among these. A subset of 56 genes were differentially expressed in the gonad, in both treatment comparisons; this overlap is more than expected by chance (Fisher's exact test, $p < .0001$; Table S7). This subset of genes, along with the other sets of differentially expressed genes, was not significantly enriched for any biological processes.

In comparing patterns of expression between brain and gonad, we find that most of the gonadal genes differentially expressed between treatments are also expressed at detectable levels in the brain; however, they are not differentially expressed in the brain (Figure S1). Tissues also differ in their among-individual variability in gene expression. Specifically, the gonad has a higher standard

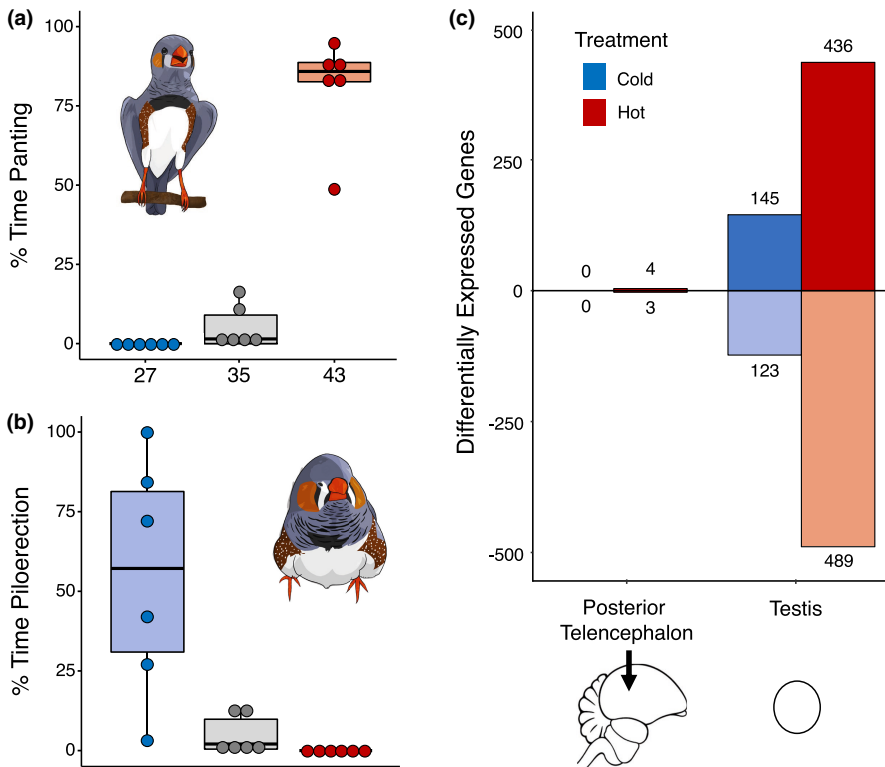


FIGURE 1 Temperature treatments affected thermoregulatory behaviours, including (a) panting and (b) piloerection, illustrated here as boxplots. Treatments also affected (c) up- and down-regulation of gene expression in the posterior telencephalon (image adapted from Nottebohm, 2005) and the testis. Bird illustrations by Mae Berlow

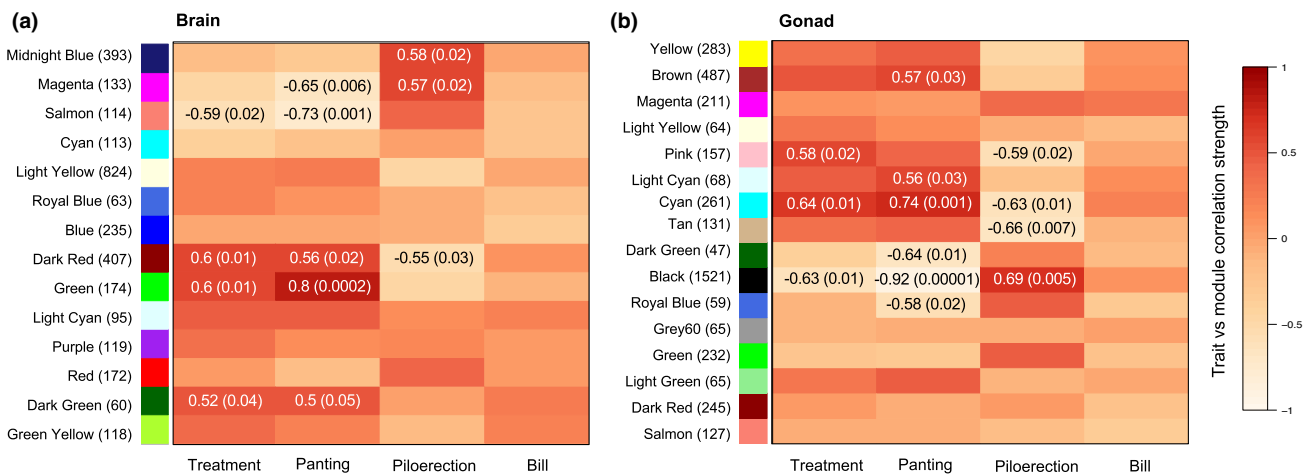


FIGURE 2 Module–trait correlations from weighted gene co-expression network analyses for (a) brain and (b) gonad samples from individuals in the hot–Thermonutral zone (TNZ) temperature contrasts. Modules are indicated by colour, and the number of genes per module is given in parentheses. For each module eigengene, heatmaps present correlation coefficients and associated p -values in parentheses for $p \leq .05$ for traits of interest. The cold–TNZ contrasts did not significantly affect any modules in either tissue, and are shown in Figure S5

error in expression differences between treatments than does the brain (Figure S3).

3.3 | Heat effects on gene networks in the brain

For the posterior telencephalon hot vs. TNZ contrast, WGCNA identified 14 modules, four of which were significantly affected by heat. Gene expression within these same four modules was also correlated

with the degree of panting (Figure 2a; Figure S4 and Table S8), and no modules correlated with treatment without also covarying with behaviour. We focus our analyses on the green and dark red modules because these two were also enriched for specific biological processes (see Tables S9 for details). Both modules included many genes with known connections to learning, production or maintenance of bird song, as detailed below.

The green module's eigengene was upregulated in response to heat, and positively correlated with treatment and panting (Figure 2a;

FIGURE 3 Functional analyses of trait-associated modules and their relationship with thermoregulatory behaviour. We show the top four GO terms for each module, based on the $-\log_{10}$ of the p -value, corrected for false discovery rate (FDR). In the brain hot vs. thermoneutral zone (TNZ) contrast, the (a) green module and (b) dark red module eigengenes correlated positively with panting and treatment. In the gonad hot-TNZ contrast, the (c) black module eigengene correlated negatively with panting and treatment. In the brain cold-TNZ contrast, the (d) magenta module eigengene correlated negatively with piloerection. For each of these modules, scatterplots depict normalized counts for a representative gene with high module membership for hot (red), TNZ (grey) and cold (blue) treatments, including (a) DRD2, which encodes a dopamine receptor and correlates with panting ($r = .69$, $p = .012$), (b) FOXP1, which encodes a transcription factor that regulates song learning and correlates with panting ($r = .68$, $p = .014$), (c) SPAG7, which encodes a gene related to spermatogenesis and correlates with panting ($r = -.86$, $p = .0008$) and (d) HSP90AA1, which encodes a heat shock protein and correlates with piloerection ($r = -.57$, $p = .054$)

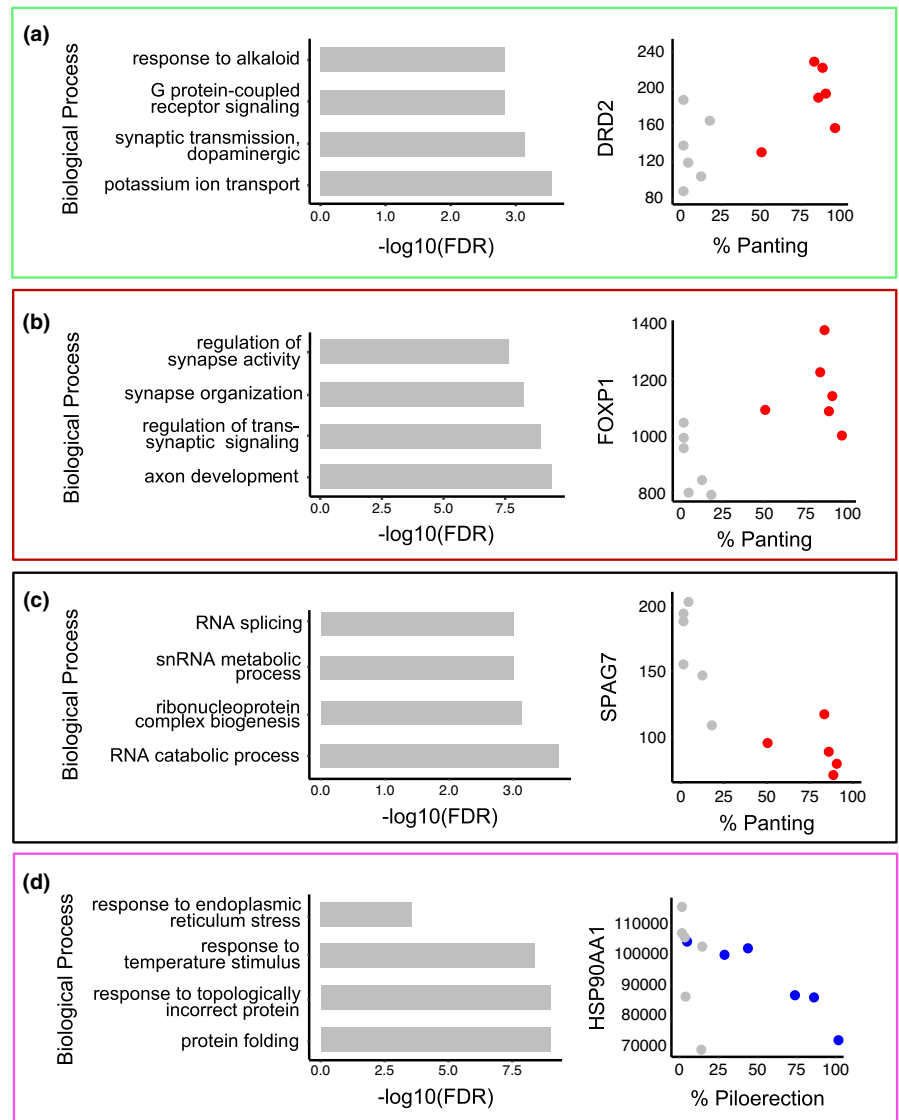


Figure S4). This module was enriched for potassium ion transport, dopaminergic synaptic transmission, G protein-coupled receptor signalling and response to alkaloid (Figure 3a; Table S9). Genes with high module membership included CHRM4 (cholinergic receptor muscarinic 4), which encodes a G protein-coupled receptor that binds acetylcholine, ADCY5 (adenylate cyclase 5), DRD2 (dopamine receptor D2) and DRD5 (Dopamine receptor D5), as well as ADORA2A (adenosine A2a receptor) and HTR1B (5-hydroxytryptamine), which encodes a serotonin receptor (Table S8). Individuals that panted more had higher expression of genes in the green module, as depicted via the example with DRD2 in Figure 3a.

The dark red module's eigengene correlated positively with treatment and panting, and correlated negatively with piloerection (Figure 2a; Figure S4). This module was enriched for axon development, regulation of trans-synaptic signalling, synapse organization and regulation of synapse activity (Figure 3b; Table S9). Genes in this module included FOXP1 (forkhead box transcription factor), LRRTM4 (leucine rich repeat transmembrane neuronal), SCN8A (sodium voltage-gated channel), GRIN1 (glutamate ionotropic receptor

NMDA), CAMK2A/B (calcium/calmodulin dependent protein kinases), CLSTN1/3 (calsyntenins), CDH4 (cadherin), NCOR2 (nuclear receptor corepressor), ROBO1 (roundabout guidance receptor) and DAGLA (diacylglycerol lipase alpha) (Table S8). Individuals that panted more also had higher expression of genes in the dark red module, as depicted by the example with FOXP1 in Figure 3b. ATP2A2, one of seven genes differentially expressed in the brain between hot and TNZ, had high network connectivity in the dark red module. This module also included two heat shock proteins, HSPA2 and HSPA8, though these did not have high module connectivity (Table S8).

3.4 | Heat effects on gene networks in the testis

In the gonad hot-TNZ contrast, WGCNA identified 16 modules, three of which were differentially regulated in relation to the treatment, and roughly half of which correlated with the degree of thermoregulatory behaviour (Figure 2b; Table S10). None of these

modules were correlated with treatment-only; most were correlated with both treatment and behaviour.

Genes within the black module were downregulated in individuals that panted more frequently and upregulated in individuals that piloerected more frequently (Figure 2b; Figure S4). The black module was enriched for RNA catabolism, ribonucleoprotein complex biogenesis, snRNA metabolic process, RNA splicing and mRNA processing (Table S9). This module included several high-connectivity genes that were also differentially expressed, including MRPL17 (mitochondrial ribosomal protein L17), RBM4 (RNA binding motif protein), TPP1 (tripeptidyl peptidase), RPUSD3 (RNA pseudouridine synthase) and UPF1 (up-frameshift mutation 1 RNA helicase and ATPase). Genes included in the black module also related to sperm function, including SPAG7 (sperm associated antigen 7) (Figure 3c) as well as PCSK4 (proprotein convertase subtilisin/kexin type 4), ZMIZ2 (zinc finger MIZ-type containing 2) and TMEM203 (transmembrane protein 203). The black module also contained several heat shock proteins, HSPB1, HSPB8, DNAJB2 and DNAJB13, though these genes were not among the highest module connectivity (Table S10).

Genes in the pink module, on the other hand, were upregulated in the testis of heat-exposed birds and downregulated among birds that displayed more frequent piloerection (Figure 2b). The pink module was enriched for chaperone-mediated protein complex assembly (Table S9) and contained genes encoding heat shock proteins including DNAJC3, which had the highest module connectivity, as well as HSPA2, HSP90AA1, DNAJC3 and AHSA2P (Table S10).

The dark green module correlated negatively with panting and was enriched for gliogenesis and regulation of apoptotic signalling (Table S9), but it did not significantly differ in relation to treatment per se.

3.5 | Cold effects on gene networks in the brain

For the cold-TNZ contrast of the brain, WGCNA identified 10 modules, but none were associated with treatment (Figure S5). The magenta module correlated negatively with piloerection ($r = -.49$, $p = .05$, Figure S4) and contained many heat shock proteins (e.g., AHSA1, AHSA2P, BAG3, DNAJA4, HSP90AA1, HSP90B1, HSPA2, HSPA5, HSPA8, HSPH1; Table S11), which had lower expression in cold compared with TNZ. The magenta model was significantly enriched for biological processes related to protein folding, as well as responses to topologically incorrect proteins, temperature stimulus and endoplasmic reticulum stress (Figure 3d; Table S9). One module (turquoise) was negatively correlated with bill surface area ($r = -.7$, $p = .003$; Figure S5), and in fact, this was the only module across all tissue contrasts that showed any significant relationship with bill surface area. The turquoise module was enriched for response to type 1 interferon (Table S9), and the gene with the highest module membership was IFI6 (interferon alpha inducible protein 6), which is involved in response to thermal stress in ants (Perez et al., 2021).

3.6 | Cold effects on gene networks in the testis

WGCNA identified 13 modules in the gonad cold vs. TNZ contrast. However, none of these were related to treatment or to any other traits of interest (Figure S5; Table S12).

4 | DISCUSSION

Behavioural thermoregulation is the first line of defence in surviving extreme temperatures (Angilletta, 2009), but how behaviour modulates the gene regulatory effects of thermal challenges is not well understood, despite potential downstream impacts on mating effort. Here, we take a transcriptome-wide approach to ask how an endotherm responds to an acute thermal challenge, focusing on neural and peripheral tissues critical to mating effort in zebra finches. We find that individual differences in behavioural thermoregulation covary with molecular pathways encoding dopamine receptors and synaptic plasticity in the brain, and spermatogenesis in the gonad. We also find that hundreds more genes are differentially expressed in the testes than in the brain, suggesting that neural tissue may be less responsive to temperature than gonadal tissue. Though mRNA levels are one aspect of molecular physiology that does not directly assess function, gene expression broadly predicts translation (Lu et al., 2007; Li et al., 2014; Mayfield et al., 2016), suggesting that patterns of gene expression can be linked to functional outcomes. Therefore, our results may shed light on the relationship between heat and mating effort.

4.1 | Panting in heat is associated with gene networks in the song control system in the brain

In the posterior telencephalon, gene network analyses reveal how thermal challenges may shape the expression of a sexually selected trait: song. Heat-exposed birds that panted more had higher expression of a suite of behaviourally relevant genes in two heat-sensitive gene networks in the brain: the green and dark red modules. In the green module, genes encoded receptors related to dopamine, serotonin and acetylcholine signalling. These systems are broadly involved in modulation of locomotion, reward, reinforcement, memory and learning (Wise, 2004), including learning and production of bird song (Asogwa et al., 2018; Heimovics & Ritters, 2008; Kubikova & Košťál, 2010; Yip et al., 2020). Our results extend previous connections between heat and dopamine, serotonin and acetylcholine signalling (e.g., Kim et al., 2013; Ray et al., 2011; Shibasaki et al., 2002), by relating the degree of behavioural thermoregulation to variation in heat-induced gene regulation in the brain. This module eigengene was not only related to treatment (Figure S4a), but there was also an intriguing positive relationship between module gene expression and the degree of thermoregulatory behaviour (Figure S4b), suggesting these effects may stem from varying degrees of behavioural thermoregulation.

In the dark red module, we identified additional heat-sensitive gene networks with strong ties to the avian song-control system (Brenowitz & Larson, 2015). This module included many genes important in song learning, including FOXP1, which regulates memory formation (Garcia-Oscos et al., 2021), LRRTM4 and CAMK2A/B, which regulate synaptic development and plasticity (Sinha et al., 2020), CLSTN1/3 and CDH4, which regulate song development (Kato & Okanoya, 2010; Matsunaga et al., 2011), and DAGLA, which is involved in adult neurogenesis (Oudin et al., 2011) and correlates with singing in European starlings (Hahn et al., 2017). GRIN1 was also found in this module and has been connected to synaptic plasticity and climate-related natural variation in memory in chickadees (Pravosudov et al., 2013). Genes also included those important in song neural circuitry, including SCN8A, which is involved in neuronal excitability (Friedrich et al., 2019) and ROBO1, which is involved in connectivity of vocal learning circuits (Wang, Chen, et al., 2015). Coupled with our previous findings that heat reduces cognitive performance (Danner et al., 2021) and song production (Coomes & Derryberry, 2021), these results provide a potential mechanistic link between heat effects on neuronal signalling and motivation-related behaviours, suggesting that one avenue by which heat alters singing is via effects on the song control areas of the brain.

4.2 | Thermoregulatory behaviours also vary with gonadal responses to heat

In the testis, the differential expression and gene-network analyses reveal how heat may affect both self-maintenance and fertility. Heat-sensitive gene expression was significantly enriched for cell morphogenesis and cell projection organization, both of which are important processes in spermatogenesis (Laiho et al., 2013; Salas-Huetos et al., 2014). Several genes in the black module correlated negatively with panting and were associated with sperm function, including PCSK4, which is critical for fertilization; ZMIZ2, which increases the transcriptional activity of androgen receptor; and TMEM203, which regulates calcium homeostasis during spermatogenesis (Shambharkar et al., 2015). Further, in birds that panted more, genes in the black and dark green modules were downregulated, with key processes related to RNA processing as well as apoptosis. The direction of these changes suggests that heat dissipation behaviour is associated with reduced apoptosis, though further study is needed to determine if these effects are beneficial or maladaptive under thermal extremes. Similar pathways are also enriched in sea urchins (Runcie et al., 2012) and sea turtles (Bentley et al., 2017) exposed to high temperature. Likewise, zebra finch nestlings exposed to high temperatures had reduced mitochondrial ATP production efficiency, though prenatal acoustic programming mitigated these effects (Udino et al., 2021). Genes in the pink module were upregulated in response to heat and enriched for chaperone protein activity, and heat shock proteins were found in both the black and pink modules. These results support two hypothesized avenues of heat effects on mating effort, via shifted investment in self-maintenance and fertility (e.g., spermatogenesis).

4.3 | Neural tissue responds less strongly than gonadal tissue

In response to both high and low temperature, we found a less dramatic pattern of differential expression in the brain as compared to the gonad. Compared to the testis, the posterior telencephalon showed two orders of magnitude fewer differentially expressed genes (Figure 1). Whereas the gonad expressed more tissue-specific genes than the brain, these two tissues shared the overwhelming majority of genes expressed (Figure S1). And yet, the brain and gonad shared no differentially expressed genes for either treatment comparison, suggesting these two tissues respond differently to temperature treatments. One interpretation of these differences is that gonadal tissues may be more thermally tolerant than neural tissues, if temperature-sensitive gene expression serves to protect from the deleterious effects of extreme temperature (Rivera et al., 2021). Alternatively, neural tissues may be more thermally tolerant than gonadal tissues, if constitutive gene expression is already prepared for heat (Rivera et al., 2021). Given that zebra finches have a decreased ability to selectively cool the brain as compared to other bird species (Bech & Midtgård, 1981), their neural tissues may achieve greater thermal tolerance by elevating baseline expression of thermally sensitive genes (i.e., frontloading) or reducing expression of these genes (i.e., dampening), which could facilitate heat tolerance when birds are pushed beyond their TNZ (Rivera et al., 2021). The nature of our sampling does not allow for repeated measures in the same individuals, and so we cannot assess whether lower differential expression in the brain could be the result of frontloading or dampening. Transcription can certainly change rapidly in response to external stimuli (Bukhari et al., 2017; Shpigler et al., 2017), and differential expression also may stem from selective RNA degradation (Romero et al., 2014) or polymerase pausing, the latter of which occurs in heat shock proteins (Bunch, 2017). Irrespective of the mechanism, our findings indicate that acute thermal events differentially alter mRNA levels in neural and gonadal tissues, raising the possibility that these tissues use different mechanisms to respond to extreme temperatures.

4.4 | Moving beyond heat shock proteins

Most work on molecular responses to thermal challenges has focused on the genes encoding heat shock proteins, as their role in protein folding is a well-established response to heat stress, particularly in ectotherms (Bentley et al., 2017; Feder & Hofmann, 1999; Perez et al., 2021; Runcie et al., 2012). We find that heat shock proteins do change in response to temperature; for instance, HSPA2, which is involved in male fertility in birds (Nixon et al., 2017; Scieglińska & Krawczyk, 2015), had significantly higher expression in the testis in response to heat. However, we found a relatively small number of heat shock proteins among differentially expressed genes, and we saw relatively low connectivity of heat shock proteins in thermally sensitive gene

regulatory networks, consistent with studies that have found few (Xie et al., 2018) to no (Finger Jr et al., 2018) candidate heat shock proteins upregulated under acute heat exposure in zebra finches, and highlighting the need to understand system-wide responses to heat.

4.5 | Thermal challenges: Distinct and shared responses to high and low temperatures

More broadly, our findings add to the growing literature on how animals respond to thermal challenges. We found that high and low temperatures engage largely different gene regulatory networks, despite some overlap in differentially expressed genes. In the brain, heat exposure influenced genes regulating synaptic activity, whereas cold exposure influenced genes involved in cellular stress response, such as protein folding. Finding hot- and cold-specific responses aligns with studies across a range of taxonomic groups suggesting that high and low temperatures are distinct stressors (Bellis & Denver, 2017; Sonna et al., 2002), and that variability in temperature may represent yet another selective pressure (Sheldon, 2019; Stager et al., 2021). We also found that fewer genes were differentially expressed in the cold-TNZ contrast as compared to the hot-TNZ contrast, for both the brain and the gonad, suggesting that high temperatures may impact transcriptomic responses to a greater degree than low temperatures. However, some transcriptomic responses are shared between hot and cold thermal challenges, such as ATP-related functions. We find ATP2A2, which is involved in Ca²⁺ pumps, among the only seven genes differentially expressed in the brain in our hot-TNZ contrast. We also find enriched ATPase activity in our cold-TNZ contrast in the brain, including AHSA1 and AHSA2P activators of HSP90 ATPase activity. Previous work in songbirds suggested that sarco/endoplasmic reticulum Ca²⁺ pumps and ATPase activity in pectoralis muscle are an important heat-generating mechanism in response to cold (Cheviron & Swanson, 2017; Stager et al., 2015; Stager & Cheviron, 2020). Together, these results suggest that both high and low temperatures engage ATP-related functions, further reiterating that both treatments push birds beyond thermoneutrality. These findings are building a much needed platform to understand the extent to which brains are vulnerable to a changing climate (Beltrán et al., 2021).

4.6 | Connecting gene expression to behavioural variation in a warming world

We found a number of patterns linking individual differences in behavioural thermoregulation to variation in gene expression in tissues important for mating effort. Specifically, we identify heat-sensitive pathways involved in synaptic activity in the brain and sperm-related processes in the gonad, reflecting the full gamut of mating effort for a male, from song production to attract a mate, to sperm production to fertilize her ova. These results provide insight into

previous studies by revealing potential mechanisms by which extreme heat may reduce mating effort (Coomes et al., 2019; Coomes & Derryberry, 2021; Hurley et al., 2018; Luther & Danner, 2016). These short-term responses are relevant to longer term exposure to extreme temperatures in the wild, as zebra finches show a limited ability to acclimatize (Cooper, Hurley, & Griffith, 2020; Wojciechowski et al., 2020), and our 5-h exposure mirrors the scope of afternoon heat in nature (Hurley et al., 2018). As the additive effects of heat scale beyond what can be mitigated by some, if not all, individuals, they also may contribute to the marked population decline in songbird populations over the last half-century (Rosenberg et al., 2019). Even arid-zone avifaunal communities, which include wild zebra finches, are at risk of severe declines, both from chronic, sublethal effects and from catastrophic mortality events (Albright et al., 2017; Conradie et al., 2019; McKechnie et al., 2012). Some individuals may be better prepared to face these challenges, as we find substantial among-individual variation in behavioural thermoregulation (Figure S4), despite limited variation in ambient temperature within each treatment. In fact, nearly all treatment-sensitive gene networks covaried with behaviour as well, suggesting behaviour may be an important modulator of transcriptomic responses to thermal challenges. This potential for behavioural change is consistent with a recent study documenting among-individual variation in physiological responses to extreme heat (Wojciechowski et al., 2020), suggesting that behavioural variation may be a target of the selective pressures imposed by rising temperatures. Taken together, our results suggest a potential interplay between thermal tolerance and mating success, which may have implications for sexual selection in a warming world, particularly since individuals varied in the extent of gene regulatory responses to the same environmental temperatures. Although follow-up studies are needed to evaluate these reproductive consequences directly, one exciting idea is that sexual selection for males that perform well in extreme temperatures could influence how thermal tolerance evolves, and vice versa.

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AUTHOR CONTRIBUTIONS

E.P.D. and K.A.R. conceived the study; C.R.H. and E.P.D. developed the methods and collected the data; S.E.L., A.M.B. and D.B.R. conducted the analyses with input from K.A.R.; S.E.L., K.A.R. and E.P.D. wrote the paper. The manuscript reflects the contributions and ideas of all authors.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

[dataset] Lipshutz, S. E., C. R. Howell, A. M. Buechlein, D. B. Rusch, K. A. Rosvall, E. P. Derryberry. 2022. Dataset for How thermal challenges change gene regulation in the songbird brain and gonad: implications for sexual selection in our changing world. Dryad: doi:10.5061/dryad.j9kd51cdf.

OPEN RESEARCH BADGES



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://doi.org/10.5061/dryad.j9kd51cdf>.

ORCID

Sara E. Lipshutz <https://orcid.org/0000-0002-9816-2977>

Kimberly A. Rosvall <https://orcid.org/0000-0003-3766-9624>

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