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Expression of heat shock proteins and thermal sensitivity of male fertility across six *Drosophila* species

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Abstract

Understanding the mechanisms that confer resilience to thermal stress is crucial in the context of climate change. Recently there has been increasing focus on sublethal effects of high temperatures, such as on reproduction. Male fertility is particularly sensitive to heat, and the upper thermal fertility limit (TFL) is a better predictor of species' geographical ranges than lethal limits (LT) alone. *Drosophila* fruit fly species vary in their TFL and in the magnitude of difference between TFL and LT, but what drives this variation is unknown. We hypothesized that expression of heat shock proteins (Hsps), known to play a role in both the heat stress response and spermatogenesis, may explain these species differences. We compared the effects of a short, moderate thermal shock on the expression of seven *Hsps* in the male reproductive tract vs. the rest of the body, across six drosophilid species. Patterns of expression varied across tissues and species both before and after heat shock. There is some indication that species with lower lethal limits show greater upregulation in response to heat shock in somatic tissue. There was no clear pattern of differential regulation in relation to absolute TFL, but a suggestion that species with a larger TFL-LT gap lack differential regulation in reproductive tissue. Hence, while *Hsp* expression may play a role, there are clearly other mechanisms that underlie the sensitivity of species' fertility to elevated temperatures which need to be assessed.

Keywords: heatwave, Hsp, spermatogenesis, heat shock, temperature stress

Introduction

Research across many species indicates that reproduction is highly vulnerable to thermal stress (Dougherty et al., 2024; Parratt et al., 2021; Walsh et al., 2019). Such sublethal effects will likely be critical as both average global temperatures rise and periods of extreme temperatures (heatwaves) become more common (Barriopedro et al., 2023). Fertility declines with rising temperatures are observed across taxa (Dougherty et al., 2024). Furthermore, reproductive output may not just decline but be entirely ablated with high temperature. Two studies using more than 40 *Drosophila* fruit fly species have shown great variation in the upper thermal limit for male fertility, or thermal fertility limit (TFL) (Parratt et al., 2021; van Heerwaarden & Sgro, 2021). TFLs differ between species in their absolute value and can also be up to 3 °C lower than species' lethal limit (LT), meaning males can become irreversibly sterile at temperatures that do not affect their survival (Parratt et al., 2021; van Heerwaarden & Sgro, 2021). TFLs have been found to be a better match to species' geographical distributions than comparable LTs (Parratt et al., 2021; van Heerwaarden & Sgro, 2021) and predict temperature-driven extinction risk (van Heerwaarden & Sgro, 2021), suggesting they will impact population persistence in relation to temperature and therefore overall biodiversity (Snook et al. in revision). Remarkably though, TFLs seem insensitive to selection, suggesting that adaptive evolutionary responses in TFLs may be limited (van Heerwaarden & Sgro, 2021). Therefore, understanding the mechanisms that underlie species differences in sensitivity of reproduction to temperature is critical, and there has been a suggestion that molecular or genetic markers could be used to predict animals' tolerance of heat stress (Hassan et al., 2019).

Across species, heat stress causes widespread transcriptional changes (Vihervaara et al., 2018). In the context of reproduction, transcriptomic responses to heat stress have been observed in the testes of various species, including pigs (Gòdia et al., 2019), mice (Li et al., 2009; Rizzoto et al., 2020), rats (Yadav et al., 2018), rabbits (Pei et al., 2012), chickens (Wang et al., 2015), *Drosophila* (Bodelón et al., 2023), and octopus (López-Galindo et al., 2019). These responses involve changes in expression of heat shock protein (Hsp), oxidative stress, DNA damage, apoptosis, immune-related genes, and small and micro RNAs. Therefore, an explanation for variation across species in temperature sensitivity may be to what extent the expression of stress response genes are rapidly inducible.

Hsps play a pivotal role in the evolutionarily conserved cellular responses to thermal stress and are present in both prokaryotes and eukaryotes (Morimoto, 2011). Hsps are key regulators of proteostasis [reviewed in Hu et al., (2022)]. Their functions include acting as molecular chaperones, recognizing and binding to misfolded proteins, to either refold or target them for degradation, and they are also involved in regulating the cell cycle, apoptosis and signal transduction (Hu et al., 2022). They have been described as "evolutionary capacitors" as they potentially shield or expose other genes to selection under differentially stressful conditions (Chen et al., 2018). *Hsps* are some of the most highly conserved genes structurally and

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functionally across all domains of life from archaea to plants and animals (Daugaard et al., 2007; Lindquist & Craig, 1988), but, e.g., differences in copy number or regulation may confer differences in heat tolerance. *Hsp* copy number has been implicated in the evolution of thermal tolerance, as, e.g., the more tolerant *D. virilis* has seven copies of *Hsp70*, while the less tolerant *D. lummei* has only five (Evgen'ev et al., 2004). While a critical part of thermal stress responses, misregulation of *Hsps* can be detrimental, and is linked to various cancers, e.g., (Hu et al., 2022). Indeed, upregulation of heat shock responses for an extended period is likely to be costly (Krebs & Feder, 1997a; Sørensen et al., 2003), and potentially reflects poor adaptation to the thermal environment (Ware-Gilmore et al., 2023), so plasticity (i.e., up and downregulation as appropriate to the conditions) is required. Additionally, *Hsps* are vital for male reproduction, being required for maturation and functioning of sperm (Abane & Mezger, 2010; Michaud et al., 1997). Given their role in both stress responses and reproductive function, *Hsps* are excellent candidates to explore in relation to the sensitivity of reproduction to heat stress.

The evolution of *Hsp* expression has been highlighted as critical to species' tolerance of environmental stress (Chen et al., 2018). However, while it is clear that the gonad transcriptome and specifically *Hsp* gene expression is sensitive to temperature, can this differential regulation explain broader patterns in sensitivity or robustness to heat stress? A number of *Hsps* are expressed in the reproductive system of *Drosophila* males (Takemori & Yamamoto, 2009). In *D. melanogaster* whole larvae, induction of *Hsp* expression has been directly linked to levels of thermal tolerance (Bettencourt et al., 2008) and likewise natural variation in Hsp70 expression across population relates to thermal tolerance (Krebs & Feder, 1997b) Greater transcriptomic changes in adult gonads in response to a short heat shock (60 min @ 32 °C) were observed in males than females in *D. subobscura* (Bodelón et al., 2023), aligning with the view that male fertility is more vulnerable to elevated temperatures (Iossa, 2019). Differences in temperature of induction and peak *Hsp* expression may underlie population or species differences in thermal tolerance. For example, northern and southern killifish populations differ in their critical thermal maxima and in *Hsp* expression of adult gill, liver or spleen; *Hsp90* upregulation occurs at a lower temperature in the more tolerant southern populations, but the magnitude of *Hsp70* induction is higher in the more sensitive northern population after heat stress (Fangue et al., 2006). Comparing *Drosophila* species with different thermal tolerance, Krebs (1999) found that whole body Hsp70 protein expression in both larvae and adults peaked at a higher temperature in the most tolerant species *D. mojavensis* compared to the less tolerant *D. melanogaster* and *D. simulans*. Therefore, both the onset and peak of expression of *Hsps* may relate to differences in thermal tolerance.

We sought to examine whether variation across *Drosophila* species in their tolerance to high temperature both in survival and reproduction, or gap between these (Parratt et al., 2021), is related to expression of *Hsps*. We hypothesized that (1) basal levels of *Hsp* expression are higher in more heat tolerant species, and/or (2) more heat tolerant species would more rapidly differentially regulate *Hsps*. We further hypothesized that (3) LT would be reflected in somatic tissue gene expression, whereas TFL would be related to reproductive tissue gene expression hence those without a TFL/LT gap would show similar expression patterns in both tissues.

Methods

Fly maintenance and species identity

We utilized laboratory stocks of six closely related cosmopolitan drosophilid species (Figure S1) that were used by Parratt et al. (2021). These species were chosen as they have diverse responses to thermal shock (Figure 1), despite shared broad latitudinal ecological range, in addition to having readily available published genomes, facilitating target gene identification and primer design.

Flies were maintained as per Parratt et al. (2021) at 23 °C in ambient humidity and a 12:12 hr light: dark cycle. Species were raised on one of four food types to minimize effects of nutritional stress (Parratt et al., 2021) (see supplementary methods) and transferred to new vials weekly. Adults for experimentation were collected from vials in which five males and five females were permitted to mate and lay eggs for 48 hr, then the larvae were left to develop fully at 23 °C. Adults were sexed as virgins within 8 hr of eclosion on ice anaesthesia and kept in single species and single sex groups of 10 until experimentation.

Heat stress exposure

There is no agreed standardized way to measure heat responses (Bretman et al., 2024; Dougherty et al., 2024), and heat stress likely reflects a combination of temperature and exposure duration (Ørsted et al., 2024; Rezende et al., 2014). We chose to capture rapid responses in gene expression simulating a situation where the temperature climbs towards the high point of the day during a heatwave. Hence, we chose a high temperature of 29 °C as this surpasses the ambient temperature experienced within the laboratory, but is well within the reproductive and survival limits by at least 5 °C for all six species (Parratt et al., 2021; van Heerwaarden & Sgro, 2021). By using this approach, we avoided by a substantial margin the risk of including dead/dying flies in our gene expression samples. At 7 days post eclosion (14 days for *D. hydei* to account for this species' delayed male sexual maturation), males were exposed to 23 or 29 °C for 30 min in an incubator to capture initial gene expression changes (Lerman et al., 2003). Given that this did not reach the published TFL temperatures, we also performed a fertility assay to assess how 29 °C affected reproductive output, again following Parratt et al. (2021).

Gene expression analysis

Whole flies were then flash frozen in liquid nitrogen and stored at −70 °C. Samples were divided into the reproductive tract (comprising the testes, accessory glands, and ejaculatory bulb) and rest of the carcass on ice. Samples were then stored in groups of five pairs of reproductive tracts/carcasses at −70 °C. For each species and both temperature treatments, six biological replicates were used.

Detailed methods for RNA extraction and cDNA synthesis, choice of reference genes, genes of interest, primer design and quantitative PCR (qPCR) are in the Supplementary Material. The expression of seven Hsps was analysed: *Hsp23*, *Hsp26*, *Hsp27*, *Hsp60A*, *Hsp60B*, *Hsp70Ba*, and *Hsp83*, as all of these are expressed in the reproductive tract of male *D.*

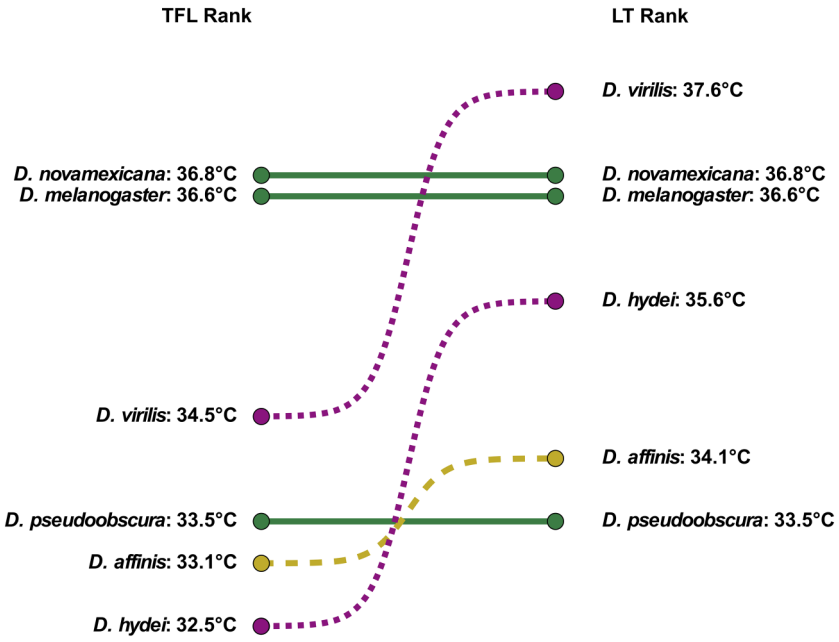

Figure 1. Comparative ranking of *Drosophila* species by TFL_{80} (thermal fertility limit defined by 80% sterility) and LT_{80} (lethal temperature defined by 80% fatality). Values taken from Parratt et al. (2021). Dashed lines indicate species with significantly lower TFL_{80} than LT_{80} and size of differences highlighted by colour—no difference (solid green line), 1 °C difference (dashed yellow line), and 3 °C difference (dotted purple line).

melanogaster (Jagla et al., 2018; Michaud et al., 1997). We then confirmed all other species had homologues of these genes (Supplementary Material S.1.21). We tested a panel of potential reference genes that are highly stable in the male reproductive system in *D. melanogaster*; actin (*Act5c*), α-Tubulin, elongation factor 1 (*EF1*), Ribosomal protein L32 (*RpL32*), TATA-Box Binding Protein (*Tbp*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and Ribophorin II (*Rpn2*) (Jagla et al., 2018; Y. Kim et al., 2019; Kofler et al., 2011; Liao et al., 2018; Matta et al., 2011; Michaud et al., 1997; Palmieri et al., 2014; Ponton et al., 2011; Zhai et al., 2014). The stability of reference genes was compared using both the carcass and male reproductive tract under 23 and 29 °C across all six species. Reference gene analysis was performed using Normfinder (Andersen et al., 2004) in addition to bestKeeper and geNorm2 utilizing the "ctrlGene" package in *R* (Zhong, 2019) to rank the reference genes across species. The two highest ranked reference genes for each species/tissue were utilized to normalize qPCR analysis, hence, we do not compare across species in a quantitative manner. Quantification of relative gene expression relative to the two best reference genes was performed using the Pfaffl method (Hellemans et al., 2007; Pfaffl, 2001). The *Ct* values for the samples were determined relative to the minimum value among the biological replicates, considering the efficiency of the primer in use.

Fertility assay

For the fertility assay, flies were heat shocked for 4 hr (starting 1 hr after lights on) following Parratt et al. (2021). Flies were allowed to recover overnight before being paired with an age-matched virgin female and allowed 24 hr to mate and lay eggs. The adults were then discarded, and the offspring were allowed to complete development at 23 °C, after which they were frozen and counted. This gave both a measure of TFL (proportion of sterile males) and of the thermal sensitivity of fertility (number of offspring) (Bretman et al., 2024) as a result of a heat shock in the adult stage.

Statistical analysis

All statistical analysis was performed using *R* version 4.2.3 (R Core Team, 2023), with each species analysed separately. We used GLMs to compare the effect of temperature on somatic/reproduction tissue for each gene, hence there are 42 models (6 species × 7 genes). We have detailed statistical analysis and figures for each comparison (by gene, species, and tissue) in the Supplementary Material (Tables S5–S11 and Figures S3–S9). Relative gene expression was determined by dividing the target gene's expression by the geometric mean of the reference genes for each sample. Gene expression fold changes were compared per gene using a linear model with Quasipoisson distribution, with tissue and temperature as fixed factors. Prior to this, a standard Poisson model was assessed for dispersion. To simplify the full model, analysis of deviances (AOD) was employed, utilizing F or χ^2 tests as appropriate. The AOD procedure aimed to retain the model's descriptive power while eliminating non-significant terms, starting with the full model with interaction terms. Once the model selection process was completed, the selected model was compared to the null model using AOD. Post-hoc pairwise comparisons between groups were performed using Tukey's tests, using the "emmeans" package (Lenth, 2022). Differential gene expression in each species between body parts at the benign temperature was performed with Mann–Whitney U tests. Within each species, the effect on temperature on the proportion of males that were infertile was analysed by χ^2 tests (with a continuity correction) and number of offspring (retaining those that produced 0 offspring) using Mann–Whitney U tests.

Results

A mild heat shock causes Hsp expression changes in somatic and reproductive tissue

Patterns of *Hsp* expression, and whether this was altered by higher temperature (i.e., differential expression), varied between species and somatic/reproductive tissue, (summarized in Figure 2 and given separately for each species/tissue in the Supplementary Material). Every species showed expression changes in relation to temperature in at least two genes, but no single gene showed differential expression in all species. There were 21/42 instances of an interaction between temperature and tissue type, i.e., that the effect of temperature differed between tissues. Where *post hoc* tests identified a significant effect in one or other tissue, this can be seen in Figure 2 (indicated by *). However, there were also five instances where the *post hoc* tests did not identify significant effects (*Hsp60A* in *D. hydei* and *D. novamexicana*, *Hsp60B* in *D. melanogaster* and *Hsp83* in *D. pseudoobscura*). There were a further seven instances of a main effect of temperature (indicated by ~ in Figure 2), hence both tissues responded in the same way to temperature.

The arrangement of species in the summary Figure 2 is to aid qualitative assessment of the relationship between *Hsp* expression and either lethal temperature (LT), TFL or the gap between these. We hypothesized that more heat tolerant species might more quickly upregulate their *Hsp* genes, and that expression in the somatic tissue (carcass) might relate to LT whereas expression in the reproductive tract might relate to TFL. There were more instances of differential expression in the carcass (20) than the reproductive tract (11) in response to temperature. In the carcass, *Hsp23*, *Hsp26*, *Hsp27*, and *Hsp70ba* were differentially expressed in 4/6 species, but none in all six species. There seems to be a qualitative relationship between the species' LT and increased *Hsp* expression, with species with lower LT showing more upregulation, and *D. virilis* (with the highest LT) showing some down regulation.

Differential expression in relation to temperature was observed less often in the reproductive tract, but *Hsp23*, *Hsp27*, and *Hsp70ba* expression increased in at least 2/6 species (Figure 2B). In *D. virilis*, the most heat tolerant in terms of LT but having one of the largest TFL-LT gaps, showed no differential expression in the reproductive tract despite 5/7 genes responding in the carcass. Similarly, *D. hydei* shows one of the largest TFL-LT gaps, and while 3 *Hsps* were upregulated in the carcass, none were in the reproductive tract. Interestingly, *D. affinis* showed the greatest response in the reproductive tract, with 5/7 *Hsps* differentially expressed, but is one of the more sensitive to heat in terms of fertility (more sterility and fewer offspring), both in our data (Figure 3) and previously (Parratt et al., 2021).

We further hypothesized that sensitivity to changes in temperature may align with the standing expression of *Hsps* prior to heat shock. At 23 °C, there were 21/42 instances of differential expression between tissues (Figure 2C). In general, *Hsps* were upregulated in the reproductive tract compared to the carcass, with the notable exception of largely downregulation in the reproductive tract of *D. virilis*. However, while at least 5/7 *Hsps* had increased expression in the most robust species, *D. novamexicana* and *D. melanogaster*, one of the most temperature sensitive, *D. affinis*, also showed differential expression of 4 *Hsps*. Only *Hsp23* showed the predicted pattern of differential expression in the more robust species and not in the more sensitive species.

Exposure to a mild heat shock can alter reproduction

All males survived 4h exposure to 29 °C, indicating as predicted this is not close to a lethal temperature for any species. Both *D. affinis* ($\chi^2_1 = 15.477$, $p < 0.001$) and *D. novamexicana* ($\chi^2_1 = 6.260$, $p = 0.022$) showed an increase in the percentage of males that were infertile at 29 °C compared to 23 °C, whereas other species showed no difference (*D. hydei* $\chi^2_1 = 1.736$, $p = 0.188$; *D. melanogaster* $\chi^2_1 = 0$, $p = 0.985$; *D. pseudoobscura* $\chi^2_1 = 0.133$, $p = 0.715$, *D. virilis* $\chi^2_1 = 2.134$, $p = 0.144$). However, at an increase of 29% and 13%, respectively, this did not approach the 80% infertile males for the TFL_{80} measure (Figure 3; Figure S10). In terms of the number of offspring produced, *D. melanogaster* showed a significant increase at 29 °C ($U = 5{,}647$, $N = 192$, $p = 0.007$), whereas *D. affinis* ($U = 3519$, $N = 199$, $p < 0.001$) showed a significant decrease (Figure 3; Figure S10). The other species showed no difference; *D. novamexicana* ($U = 4{,}509.5$, $N = 195$, $p = 0.541$), *D. pseudoosbcura* ($U = 4{,}577.5$, $N = 197$, $p = 0.490$), *D. hydei* ($U = 4{,}203$, $N = 189$, $p = 0.487$), and *D. virilis* ($U = 5{,}105$, $N = 193$, $p = 0.245$).

Discussion

We hypothesized that differences in standing expression or ability to rapidly induce *Hsps* would relate to thermal sensitivity variation across *Drosophila* species, specifically to the absolute LT, TFL, or the disparity between LT and TFL. We found some evidence for these ideas, but the overall picture is more complex. There was some relationship between LT and expression responses to increased temperature, where more sensitive species showed broader expression changes, but not with absolute TFL. Species that showed the largest gap between LT and TFL, *D. virilis* and *D. hydei*, showed some significant thermal regulation of *Hsps* in somatic but not in reproductive tissues. At a benign temperature, *Hsp* expression was largely higher in the reproductive tract compared to the carcass, and especially in *D. novamexicana* and *D. melanogaster*, the two species with the highest absolute TFL.

We found that even a mild, short thermal stress that is not close to lethal limits can induce *Hsp* expression changes, suggesting these are very dynamic responses to stress. In relation to expression in somatic tissue, the species with the lowest LT (*D. pseudoobscura* and *D. affinis*) showed the greatest increase in expression, whereas the species with the highest LT (*D. virilis*) showed decreases in expression. Interestingly, heat-hardening (a period of increased temperature before an extreme heat shock) can improve *D. virilis* survival but not fertility (Walsh et al., 2021), and we may have captured part of that process here. The finding that increased expression occurs in more sensitive species at lower temperatures is in line with a previous comparison across three *Drosophila* species of Hsp70 expression (through ELISA rather than qPCR), where the more sensitive species (*D. melanogaster* and *D. simulans*) showed higher peak expression at a lower temperature than the more tolerant species (*D. mojavensis*) (Krebs, 1999). Hence, rather than supporting our hypothesis that species which are more thermally robust can upregulate *Hsp* genes more rapidly, overall this suggests that species differences in

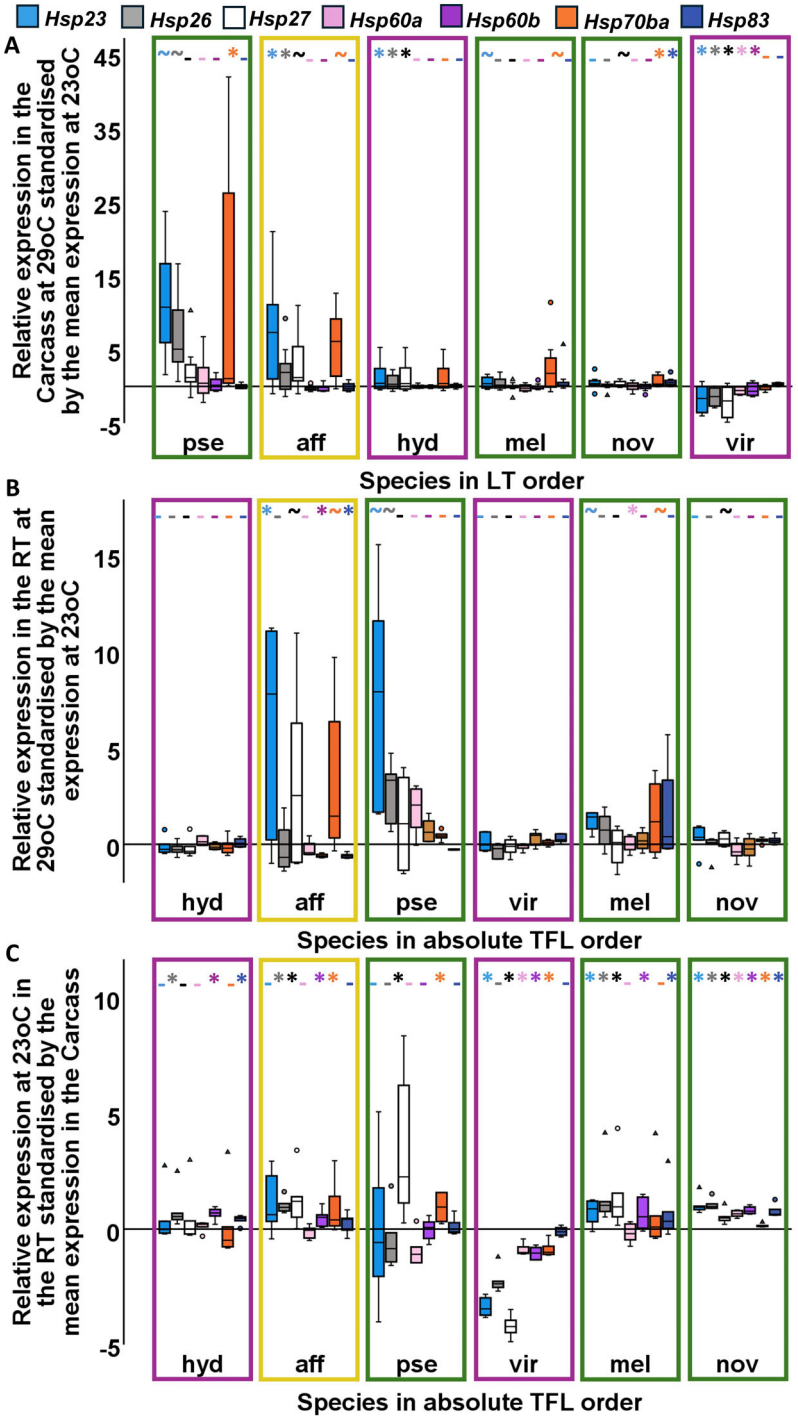

Figure 2. Summary of relative expression (RE) patterns of seven *Hsp* genes in six *Drosophila* species; *D. hydei* (hyd), *D. affinis* (aff), *D. pseudoobscura* (pse), *D. virilis* (vir), *D. melanogaster* (mel), and *D. novamexicana* (nov). Flies were kept at 23 °C or exposed to 29 °C for 30 min prior to tissue collection. For illustration, each value has been standardized by the mean RE of their comparison treatment, calculated by (sample RE—[mean comparison RE]). Hence, a value of 0 means the RE for that sample = the control mean, values above/below 0 suggest up/down regulation. (A) The carcass only RE at 29 °C standardized by the mean of 23 °C, (B) reproductive tract (RT) only RE at 29 °C standardized by the mean of 23 °C, and (C) for RE at 23 °C, the RT standardized by the carcass mean. In (A), species are ordered from lowest to highest lethal temperature (LT) and in B/C from lowest to highest absolute TFL. Species difference between TFL_{80} and LT_{80} are highlighted by the coloured border—no difference (green), 1 °C difference (yellow), and 3 °C difference (purple). Boxplots represent median and Interquartile Range of relative expression. Outliers, if any, are indicated by circles or triangles outside the whiskers. The outcomes of statistical tests are illustrated across the top of the panels; a—representing no significant effect. In (A) and (B), Generalised Linear Models (GLMs) were used to assess the interaction between temperature and tissue, where there was a significant interaction * indicates that a pairwise post hoc test within tissue revealed a significant effect of temperature, whereas where there was no significant interaction, ~ indicates a main effect of temperature. In (C), * indicates a significant difference between carcass and reproductive tract at 23 °C analysed using Mann Whitney U (MWU) tests.

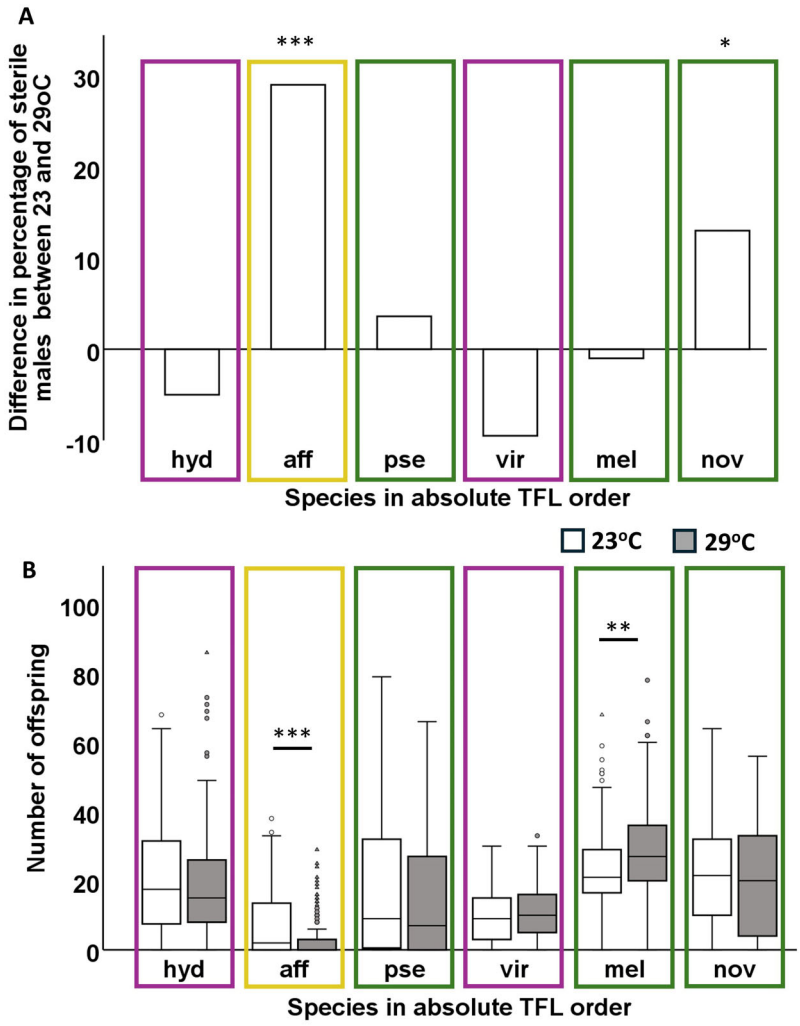

Figure 3. Reproductive output in relation to increased temperature in six *Drosophila* species; *D. hydei* (hyd), *D. affinis* (aff), *D. pseudoobscura* (pse), *D. virilis* (vir), *D. melanogaster* (mel), and *D. novamexicana* (nov). Flies were either kept at 23 °C (white) or exposed to 29 °C (grey) for 4 hr and left to recover prior to exposure to a virgin female for 24 hr. Reproductive output is illustrated as (A) the difference in percentage of sterile males between temperatures calculated by (% sterile in 29 °C treatment) − (% sterile in 23 °C treatment) (B) number of progeny produced within 24 hr. Species difference between TFL_{80} and LT_{80} identified and highlighted by coloured border—no difference (green), 1 °C difference (yellow), and 3 °C difference (purple). Boxplots represent median and Inter Quartile Range, outliers are represented by circles/triangles. Final sample sizes 23 °C treatment/29 °C treatment; hyd = 96/93, aff = 92/107, pse = 99/98, vir = 97/96, mel = 95/97, and nov = 100/95. Asterisks indicate significant differences $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

induction relate to how close the thermal shock is to their thermal maxima.

We found that expression of *Hsp* genes in the reproductive tract can likewise be induced by a short, mild thermal shock. However, while *D. affinis*, the most sensitive species in our fertility assays, showed the largest number of changes in *Hsp* genes in the reproductive tract, overall expression patterns did not seem to relate to the species' absolute TFL. Moreover, the same genes were not necessarily active in both somatic and reproductive tract, suggesting responses or protective mechanisms are different in these tissues. Perhaps the most striking examples of this are *D. virilis* and *D. hydei*, the species here with the greatest TFL-LT gap at 3 °C. Both these species showed some differential expression of specific *Hsps* in their carcass, but did not show any in their reproductive tract. This difference in expression activity between tissues may therefore go some way to explaining the TFL-LT gap. *Hsp23* was upregulated in the reproductive tract of the other four species, and *Hsp27* in three of these species, so these may be the best candidates for further functional analysis. We had also hypothesized that the standing expression of *Hsp* genes primed species to withstand thermal shocks. Across species, we found a general trend for increased expression of *Hsps* in the reproductive tract at benign temperatures, again with *Hsp23* being more highly expressed in the more robust species and not in the most sensitive. *Drosophila virilis* stands out as having generally lower expression in the RT compared to the carcass, but as this was not mirrored in *D. hydei*, and is therefore unlikely to explain the TFL-LT gap. Hence, while there may be some role for *Hsp* expression underlying the variation in thermal sensitivity, other mechanisms must also be at play.

We chose genes of interest based on their established link to both thermal stress and reproduction, however very few studies have assessed their role in reproductive responses to heat. For example, in chicken testes after a 4 hr shock at 38 °C, HSP25, HSP70, and HSP90AA1 were upregulated (Wang et

al., 2013). In the minnow *Puntius sophore*, *Hsp60*, *Hsp70*, and *Hsp90* were all downregulated in the testes after a 7 day exposure to 36 °C (Mahanty et al., 2019). There is also some evidence of seasonal variation, as in domestic bulls expression of HSP70.1, HSP70.2, HSP90, and HSP105 was higher during the summer months (Soren et al., 2018). In *D. melanogaster*, *Hsp23* and *Hsp27* expression has been observed in testes though unchanged by heat shock, whereas *Hsp70* was upregulated at higher temperatures (Michaud et al., 1997). In combination with our findings, this suggests that a functional analysis of these genes would be informative. Interestingly, knockout of *Hsp23* improves thermal tolerance of *D. melanogaster* to mildly higher temperatures (27 °C) (Gu et al., 2021), highlighting that upregulation may not always confer increased resilience to a thermal challenge. This study also found a slightly positive effect of *Hsp23* knockout on fecundity, though it is unclear if this was a male or female effect (Gu et al., 2021).

We chose to employ a mild stress temperature consistent across all species to capture early gene expression responses to increasing temperature without severely impacting survivorship. Assessing gene expression at the absolute limits may have impacted our analysis because we would not have known, e.g., if flies were inactive or dead when sampled. However, our approach of using a consistent temperature meant this temperature stress was closer to the LT/TFL of more heat sensitive species. Only *D. affinis* was observed to suffer a decrease in fertility (and therefore offspring output) at 29 °C, hence it may be that this was not sufficiently stressful for most species to capture the peak expression changes of *Hsps*. *Drosophila melanogaster* actually increased its progeny following exposure to 29 °C, while previous findings show that larvae exposed to this temperature suffer a decline in their number of offspring (Chakir et al., 2002; Canal Domenech and Fricke, 2022), reiterating that heat stress at different life stages can have quite different outcomes. We also found that *D. novamexicana*, reported as the most robust in terms of TFL (Parratt et al., 2021), suffered some increased infertility under mild stress. It may be then that it is more informative both to measure species' gene expression relative to their own LT/TFL, and at a more stressful temperature (e.g., to 1 °C of their limits rather than 5 °C), to better capture the variation in responses.

We note that we only allowed males one mating opportunity and measured fertility/fecundity for only 24 hr. In Parratt et al. (2021), males were given new virgin females 7 days after heat shock, and some species, including *D. virilis*, show a pattern of being initially fertile, but become sterile after 7 days. We did, however, use the identical stocks and maintenance regimes (diet, light regime, and control temperature) as Parratt et al. (2021). However, nutrition may introduce differences in how species respond to temperature (K. E. Kim et al., 2020) and the optimal light regime may differ between more northerly and southerly species, and gene expression is known to show strong circadian rhythm (Claridge-Chang et al., 2001). Nevertheless, our findings suggest that reproductive performance curves may vary considerably in their shape and not only in absolute values, and so it is informative to measure impacts on both fertility (TFL) and fecundity (thermal sensitivity of fertility TSF) at a range of temperatures (Bretman et al., 2024).

We acknowledge that with only six species it is difficult to discern general patterns, and given how we performed the experiments, we did not formally statistically compare across species. Moreover, with only one time point, we also may not have sampled at the appropriate time, e.g., most *Hsp70* activity, especially when activated by severe heat shock, occurs after the organism has returned to physiologically normal temperatures (Krebs and Feder, 1997a). While we focussed on *Hsps*, clearly there are other genes, RNAs and transposable elements important under thermal stress that could be revealed by differential expression analysis (Bodelón et al., 2023) and so a screen of whole transcriptome responses would be informative. Additionally, some recent studies have been using a Thermal Death Time or Thermal Landscape approach that integrates time and temperature to assess thermal sensitivity (Ørsted et al., 2024; Rezende et al., 2014), and it may be useful to employ this design to assess transcriptome changes as damage accumulates.

Overall, we have shown that *Hsp* gene expression may be related to species' tolerance to heat in terms of standing expression in more robust species, and in response to a short, moderate thermal stress. However these patterns are highly species and context dependent, so it remains to be determined whether any could act as a predictor of species' reproductive robustness.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

Data is freely available from the Research Data Leeds Repository via https://doi.org/10.5518/1736

Author contributions

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Conflict of interest

None declared.

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