Molecular mechanisms underlying plasticity in a thermally varying environment

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Abstract

Adaptation to environmental variability is a prerequisite for species' persistence in their natural environments. With climate change predicted to increase the frequency and severity of temperature fluctuations, ectothermic organisms may increasingly depend on acclimation capacity to accommodate thermal variability. To elucidate the molecular basis of fluctuating temperature-induced phenotypic plasticity, we investigated heat tolerance and the mechanisms induced by acclimation to thermal variability as compared to those seen at constant temperature. We ran genome-wide transcriptomic analysis on *Drosophila melanogaster* subjected to acclimation at constant (19 ± 0°C) and fluctuating (19 ± 8°C) temperatures and contrasted the induction of molecular mechanisms in adult males, adult females and larvae. We found life stage- and sex-specific dynamics of the acclimation responses to fluctuating temperatures. Adult flies exposed to temperature fluctuations showed a constitutive improvement in heat tolerance while heat tolerance of larvae tracked thermal fluctuations. A constitutive down-regulation of gene expression was observed for several genes in the larvae exposed to fluctuations. Our results for adult females showed that, for several genes, fluctuating temperature acclimation resulted in canalization of gene expression. Both transcriptional and post-transcriptional machinery were greatly affected by fluctuations in adult males. Gene ontology analysis showed enrichment of the heat stress response involving several major heat shock proteins in both larvae and adults exposed to fluctuating temperatures, even though fluctuations were in a benign range of temperatures. Finally, molecular mechanisms related to environmental sensing seem to be an important component of insect responses to thermal variability.

KEYWORDS

gene expression, molecular adaptation, phenotypic plasticity, thermal fluctuations, time to knock-down
1 | INTRODUCTION

Temperature is one of the most important abiotic factors affecting the performance of terrestrial ectotherms (Angilletta et al., 2002; Huey & Kingsolver, 1989). With predictions of global climate change-induced warming and increased occurrences of severe temperature fluctuations, ectothermic species are expected to be negatively affected (Foray et al., 2014; Paaljimans et al., 2013). This has turned the attention to the potential of behavioural thermoregulation or thermal acclimation to alleviate the negative effects of thermally heterogeneous environments (Grant & Dunham, 1988). The scope for behavioural thermoregulation to buffer against stressful temperatures (especially in small ectotherms) could, however, be limited by the microclimates available to different life stages of the organism (Dillon et al., 2009; Gibbs et al., 2003; Kingsolver et al., 2011). Consequently, physiological adjustments via acclimation have been suggested to play a critical role for dealing with thermal variability (Seebacher et al., 2014; Sgrò et al., 2016).

Studies investigating thermal acclimation capacity in ectotherms typically use constant temperature acclimation regimes. These studies have provided a mechanistic understanding of thermal acclimation (Angilletta, 2009; Chen et al., 2015a, 2015b; Sørensen et al., 2003), but have also shown that constant temperature acclimation may provide limited scope for accommodating the effects of stressful high temperatures (Gunderson & Stillman, 2015; Sørensen, Kristensen, et al., 2016). Moreover, environmental temperatures are rarely constant and there is a growing understanding that acclimation responses at constant temperatures might not reflect realized acclimation under ecologically relevant fluctuating temperatures (Colinet et al., 2015; Foray et al., 2014; Salachan & Sørensen, 2017). Our knowledge of the potential of acclimation capacity is further confounded by phenotypic differences in response to thermal variability observed among different life stages of an organism (Salachan & Sørensen, 2017). Thus, the focus on adult insects and on constant temperatures might only partly reflect the ecologically relevant potential for thermal acclimation.

Mechanistic studies of *Drosophila melanogaster* have shown that acclimation to thermal fluctuations induces transcriptional responses independent from the responses to constant temperature. Small heat shock proteins were up-regulated by temperature fluctuations as compared to constant temperature in *Austrofundulus limnaeus* fishes. Although responses to both constant and fluctuating temperatures in these fish involved aspects of cellular growth and proliferation, these were regulated by different genes in each case (Podrabsky & Somero, 2004). In the parasitic wasp *Aphidius colemani*, proteomic analysis suggested that cytoskeletal components could play a role in mediating beneficial acclimation responses to fluctuating temperature (Colinet et al., 2007). In adult *D. melanogaster*, the effects of fluctuation were found to be independent of the classic heat shock response (Sørensen, Schou, et al., 2016). Thus, while some candidate mechanisms exist (see also Ebner et al., 2019), the molecular basis of physiological acclimation to ecologically relevant thermal fluctuations are far from being fully understood. In particular, it is an open question whether fluctuations act by permanently activating the involved mechanisms, or whether fluctuations repeatedly activate and repress mechanisms, during the hot (or heating) and the cold (or cooling) phase (Long et al., 2012). For example, *D. melanogaster* responded to diurnally varying field temperatures by continuous adjustment of their heat and cold tolerance to the prevailing temperature (Overgaard & Sørensen, 2008). In contrast, Manenti et al. (2018) showed that heat tolerance did not track simulated diurnal temperature variation, but that constitutively improved heat tolerance accumulated across repeated fluctuations. Similarly, larvae of *Belgica antarctica* showed constitutive up-regulation of heat shock proteins and a corresponding higher intrinsic heat tolerance as compared to the adults (Rinehart et al., 2006). These results show that transcriptional responses to fluctuating temperatures vary, dependent on the life stage of the organism, the species or the exact condition of the applied experimental regime.

To improve our understanding of the thermal ecology of insects and other ectotherms, it is crucial to understand responses to fluctuating temperatures and to characterize the molecular underpinnings of these responses. Here, we investigated acclimation responses to simulated diurnal temperature fluctuations in a population of *D. melanogaster* at both the transcriptomic and phenotypic level. We subjected experimental insects to a constant (CR) or a fluctuating thermal regime (FR) and included both larvae and adult males or females to distinguish between developmental and adult acclimation effects. We exposed larvae and adults to an equal number of fluctuations (six cycles) for comparability across life stages. Furthermore, we subjected adults to one or two consecutive rounds of six thermal fluctuation cycles, to investigate the temporal dynamics of the induced molecular mechanisms during repeated fluctuations. To investigate the temporal dynamics within a thermal fluctuation, we sampled experimental subjects at the high and low temperature point of a fluctuation cycle (see Figure 1 for our experimental design). We predict that genes differing in expression levels between the fluctuating and the constant regimes explain the difference in heat tolerance and therefore are crucial for the response of *D. melanogaster* to fluctuating temperatures. Specifically, we hypothesize differentially expressed genes to fall in one of two main patterns: (i) genes are constitutively up- or down-regulated in FR relative to CR, or (ii) genes are differentially expressed (FR vs. CR) based on the fluctuating pattern across time points, i.e., are responding directly to (tracking) the temperature fluctuations.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

We used a laboratory population of *Drosophila melanogaster* collected in 2016, from Odder, Denmark (Schou et al., 2016). We used an outbred population as it represents the responses of a natural population better than an inbred laboratory strain. The flies were reared in the laboratory at 25°C (12-h:12-h light-dark) on standard...
Drosophila oatmeal–sugar–yeast–agar medium. All experimental animals were produced using density control by transferring ~40 (±3) eggs (laid at 25°C) into fresh 7-ml food vials.

2.2 Developmental and adult acclimation

Two acclimation regimes were used. These were a sinusoidal fluctuating regime with a mean of 19 and an amplitude of ±8°C and a constant regime serving as a control at 19 ±0°C. Larger amplitudes are known to induce more pronounced phenotypic effects (e.g., Kingsolver et al., 2016; Měráková & Gvoždík, 2009), and this treatment regime was earlier found to elicit a beneficial acclimation response in critical thermal maxima (CTmax) for D. melanogaster (Salachan & Sørensen, 2017). For the fluctuating regime, treatment started at 8 a.m. at the mean temperature (19°C), increased to 27°C over 6 h and decreased to 19°C over the next 6 h. A mirrored pattern was followed over the next 12 h where the temperature reached a minimum of 11°C, thus maintaining a mean temperature of 19°C over the course of a 24-h period. A mean temperature of 19°C was chosen so that fluctuations would not reach stressful temperatures, which are known to elicit different stress responses as opposed to fluctuations within nonstressful temperatures (Estay et al., 2014; Folguera et al., 2011; Kingsolver et al., 2009). This presents a limitation to this study, in that the effect of fluctuations per se cannot be distinguished from the response to reaching the high temperature point of the thermal fluctuation. The two are integral components of a fluctuation. We chose not to include constant temperature controls at 27 and 11°C, as these would have been additionally confounded by mean temperature differences. We have previously shown that fluctuations resulted in better thermal tolerance than constant treatment temperatures but not on a par with the effect of high point of temperature acclimation if it were to be applied constantly (e.g., flies reared at constant 19°C performed worse in CTmax than those at fluctuating 19 ±4°C, which in turn did worse than flies acclimated to constant 23°C acclimation) (Salachan & Sørensen, 2017).

Both regimes in the present study were maintained under 12-h:12-h light–dark photoperiodic conditions. For developmental acclimation, collected eggs were maintained at FR or CR conditions from collection of the egg to sampling at the larval stage (see below). Adult acclimation was initiated only at the adult stage (i.e., development took place at the CR condition) wherein newly emerged flies (6–12 h old) were separated by sex and maintained in 7-ml food vials in groups of 20 individuals until sampling at either the FR or the CR condition.

2.3 Thermal assay

Larvae (in the third instar stage) from both the FR and the CR regimes were sampled from the side of the vials during the high and low temperature points of the seventh fluctuation cycle (i.e., after receiving six full fluctuation cycles). These time points are hereafter referred to as High1 and Low1. We did not observe major differences in the developmental time to third instar for the FR and CR larvae. Individuals were sampled as wandering larvae, a behaviour characterizing the end of the third instar stage (Sokolowski et al., 1984). We conducted a static time to knock-down (TKD) assay following an approach differing from the more conventional water bath method (e.g., Jørgensen et al., 2019) since assaying larval TKD is not possible using this assay. In this instance, 36 larvae from each of the
two regimes, CR and FR, were individually assigned to experimental wells (6 mm high by 16 mm wide) within test arenas covered by a plexi glass lid. The arenas were then placed in an incubator (POL-EKO ILW 115 TOP+) at 41°C and filmed for 46 min using a tablet (Samsung Galaxy Tab A). Survival times were estimated as the last movement detected by visual inspection of each recording.

Adult males and females were collected during the seventh or 13th fluctuation cycle (i.e., after receiving six or 12 complete fluctuation cycles, respectively; see Figure 1 and Table S1). These time points will be referred to as High2 and Low2 (for the flies sampled during the high and low temperature points of the seventh fluctuation, respectively), and High3 and Low3 (flies sampled during the high and low temperature points of the thirteenth fluctuation, respectively). We followed the same procedure as used for larvae for assaying heat tolerance for the individual adult male and female flies as described above (36 flies ×2 regimes ×2 sex). We utilized two incubator cabinets (identical models of POL-EKO ILW 115 TOP+) to concurrently record all the samples (18 flies ×2 regimes ×2 sex per incubator).

2.4 | Analysis of phenotypic data

We investigated the effect of fluctuating temperature acclimation on larvae at various time points by performing survival analysis on the larval phenotypic data (Salachan & Sørensen, 2022) using the “survival” package (Therneau, 2015) in R (R Core Team, 2018). As we observed many larvae had survived past the 46 min of filming, we included those samples as right censored data for the analysis. We decided to go with a conservative approach and included lane (columns in the experimental arena) as a fixed categorical factor instead of as a random factor for the larval samples. Survival probabilities were estimated from a Cox proportional hazards model and were adjusted for lane effects using the “survminer” package (Kassambara et al., 2019). Regime effects were analysed by model comparisons with chi-square ($\chi^2$) statistics and $p$ values <.05 were taken to be significant.

To investigate the effect of fluctuating temperature acclimation on adults at each time point, we performed ANOVAs, comparing linear mixed effects models with sequentially decreasing model complexity using the “lme4” package (version 1.1-5) (Bates et al., 2015) in R (R Core Team, 2018) using the adult phenotypic data (Salachan & Sørensen, 2022). We included regime and sex as categorical fixed factors. Lane was included as a random factor to account for potential influences from the positioning of the flies in the arena, as was also the case for the larval assay (as we observed an influence along the horizontal axis). We report the $\chi^2$ values from the model comparisons. QQ and residual plots were used for inspecting the assumptions of a parametric test.

2.5 | RNA sequencing and bioinformatic analysis

Corresponding to each of the time points of the heat tolerance assay (Figure 1), we sampled a group of fifteen 3rd-instar larvae, 10 adult male or 10 female flies into 1.5-ml Eppendorf tubes. A total of four biological replicates were made for each acclimation regime (80 samples in total; Table S1). The tubes were immediately frozen in liquid nitrogen and stored at ~80°C until processing. RNA extraction, library construction and high-throughput RNA sequencing were done by Novogene. More than 30 million paired-end reads were obtained for each sample. The raw reads (Salachan & Sørensen, 2020) were checked for quality using FASTQC (Andrews, 2010) and trimmed based on a minimum length of 80 bp (Table S2). Alignment of the trimmed reads was done using HISAT2 with default settings (Kim et al., 2015). STRINGTIE (Pertea et al., 2015) was used to assemble the transcripts based on the D. melanogaster reference genome (BDGP release 6).

All the transcripts were further merged using the merge function of STRINGTIE and run again with the B option on the merged transcripts to produce BALLGOWN input table files. Gene counts (Salachan & Sørensen, 2020) were finally generated using a python script provided by the developers of STRINGTIE and were used for subsequent analyses (transcripts were from here on interpreted as genes, but note that no gene isoforms were analysed).

2.6 | Differential gene expression analysis

We used DESEQ2 (Love et al., 2014) to obtain differentially expressed genes between our two regimes (FR and CR) across time points (referred to as “overall” analyses). Statistical significance was based on likelihood ratio tests (LRTs) comparing a full model (time point, regime and their interaction) to a reduced model without the interaction term (i.e., just time point and regime). Genes that have a low $p$ value (Benjamini–Hochberg corrected $p < .01$, Wald statistics) from this test are those which show time point-specific differences between FR and CR at any time point after the first one. For the functional analysis, an adjusted $p$ value $< .01$ and a log$_2$ fold change $>$0.58 (up-regulated) or $<$−0.58 (down-regulated) were selected (corresponding to a fold change of 1.5).

For deriving temporal gene expression patterns, we used a regression fit for each gene followed by a stepwise regression analysis using MASIGPRO (Conesa & Nueda, 2019). A variable selection procedure was then implemented in MASIGPRO with default parameters (including cut-off value for regression coefficient of 0.6) on the differentially expressed genes (Benjamini–Hochberg corrected $p < .05$) identified by MASIGPRO. The MASIGPRO default number ($k = 9$) of expression patterns was generated for adults using k-means clustering (increasing the number of clusters did not result in additional unique and biologically meaningful patterns). A smaller number ($k = 4$) of clusters was generated for the larval data (as these data contained fewer time points and thus fewer possible patterns). Genes from each cluster were used separately for downstream functional analysis.

For all data sets we performed Gene Ontology (GO) analysis to identify enriched functional groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to obtain functional pathways (DAVID Bioinformatics Resources: da Huang et al., 2009a, 2009b, 2009c, 2009d).
we removed one replicate sample from the first low temperature point (Low2) of the adult male samples as it represented an outlier, accounting for most of the variation (data not shown).

From our “overall” analyses using DESeq2, we obtained 428 (221 up- and 207 down-regulated), 3005 (1482 up- and 1523 down-regulated) and 4042 (2041 up- and 2001 down-regulated) differentially expressed genes between constant and fluctuating regimes at an adjusted $p$ value of .01 for the larval, male and female samples, respectively (Figure 2c). Of these, 117 genes were shared between males and larvae, 140 between females and larvae, and 1567 between males and females (Figure 2c). The overlaps between the different gene sets were higher than what would have been expected by chance, based on an underlying hypergeometric distribution ($p = 3.9 \times 10^{-8}$, $1.4 \times 10^{-6}$ and $\leq 2.2 \times 10^{-16}$ for overlaps between males and larvae, females and larvae, and males and females, respectively).

Among the pairwise overlaps of the differentially expressed genes, 77 were shared among all the three groups (Figure 2c).

Using MAStipro (Conesa & Nueda, 2019), we obtained 5032 (larvae), 8742 (male) and 8104 (female) differentially expressed genes across time points at an adjusted $p$ value of .05 (Benjamini-Hochberg corrected false discovery rate [FDR]). These were used to derive common gene expression patterns based on $k$-means clustering (Figures 4–6). For larvae, three of the four patterns showed a clear change in gene expression (between up- or down-regulation) based on the time point, suggesting a clear interaction effect (patterns A, C and D; Figure 4). For the remaining pattern (pattern B; Figure 4), we observed a constitutive down-regulation of gene expression for the larvae acclimated to FR compared to the CR larvae. For adult female (Figure 5) and male (Figure 6) expression patterns, we observed constitutive up- or down-regulation of gene expression across all time points in several patterns for the flies exposed to fluctuating temperatures. However, more interestingly, for the female transcriptome, we additionally identified several patterns that were characterized by some degree of canalized (i.e., stable) gene expression in the fluctuating as compared to the constant regime (patterns A, C, E and F; Figure 5).

### 3.4 | Gene ontology and KEGG pathway analysis

We ran the DAVID functional annotation suite with default settings and extracted GO terms as well as KEGG pathways for all gene lists from our differential gene expression analyses. Significant GO terms and KEGG pathways were selected based on an FDR value <.01 (in combination with an adj. $p < .01$ used to select each gene; we consider this FDR value to be a reasonable threshold to identify significantly robust functional enrichments). The results from the analysis are summarized in Table 2 (temporal patterns) and Table 3 (overall analyses). Important pathways differentially regulated between constant and fluctuating regimes were “Protein processing in endoplasmic reticulum,” “Cytochrome P450” superfamily and “Glutathione metabolism.” Biological processes included “heat shock-mediated
polytene chromosome puffing,” “response to heat,” “response to unfolded protein,” “regulation of transcription,” “ribosome biogenesis,” “photoreceptor cell axon guidance,” “negative regulation of histone modification,” “calcium ion transmembrane transport,” “potassium ion transport” and “oxidation-reduction process.” In addition, functional analyses of the genes that were identified in the “overall” analyses, and which were identified to be differentially regulated uniquely in either males, females or larvae, are summarized in Table 4. Notably, GO terms “neurogenesis,” “negative regulation of histone modifications” and “metabolism of xenobiotics by cytochrome P450” were unique to males, females and larvae, respectively. KEGG pathway “protein processing in endoplasmic reticulum” and GO terms “response to heat” and “protein folding” were commonly enriched for all three groups.

4 | DISCUSSION

Understanding the mechanisms by which insects respond to ecologically relevant thermal conditions is crucial for advancing our knowledge on their thermal biology. So far, several studies have documented the beneficial effects of acclimation to fluctuating temperatures in insects and other ectotherms (Hutchison & Ferrance, 1970; Koštál et al., 2007; Petavy et al., 2001; Renault et al., 2004;
Figure 3. TKD estimates at 41°C for adult Drosophila melanogaster measured across time points (High2 and High3 correspond to the high temperature point, and Low2 and Low3 to the low temperature point of the 7 and 13 fluctuation, respectively) for both female and male samples. Symbols and error bars represent mean ± SEM TKD for the two regimes: constant (circle) and fluctuating (triangle) regimes. Coloured (constant: red, and fluctuating: blue) lines represent the reaction norms of each regime. N = 31, 35, 36 and 34 for females across time points and N = 33, 35, 36 and 36 for males across time points.

Table 1. ANOVA results for effect of fluctuations on TKD at various time points during adult acclimation.

| Time point | High2  | Low2  | High3  | Low3  |
|------------|--------|-------|--------|-------|
| Regime ($\chi^2_{df}$) 12.83, 136 *** | 23.85, 142 *** | 9.83, 144 ** | 11.29, 142 *** |
| Sex ($\chi^2_{df}$) 12.23, 136 *** | 8.07, 142 * | 10.57, 144 ** | 11.47, 142 *** |

Note: Time point represents the sampling points in time. High2, high point of fluctuation after six fluctuations (i.e., high temperature point of 7 fluctuation). Low2, low point of fluctuation after six fluctuations (i.e., low temperature point of 7 fluctuation). High3, high point of fluctuation after 12 fluctuations (i.e., high temperature point of 13 fluctuation). Low3, low point of fluctuation after 12 fluctuations (i.e., low temperature point of 13 fluctuation). Regime represents adult constant and fluctuating regimes and Sex is males and females.

Abbreviation: TKD, time to knock-down.

***p < .001, **p < .01.

Figure 4. Differentially expressed genes identified by MASICPRO and clustered into four expression patterns for larval samples based on k-means clustering. The x-axis corresponds to the time point in days (High1 corresponds to high and Low1 to low temperature time points of the 7 fluctuation) and y-axis to the expression value. For plotting, the time points are considered as factorial.
A few studies have investigated the mechanisms induced by fluctuating temperatures (Colinet et al., 2016; Manenti et al., 2018; Podrabsky & Somero, 2004; Sørensen, Schou, et al., 2016). However, no study has so far established the adaptive mechanisms induced by high vs. low temperatures reached during thermal fluctuations across life stages.

For the phenotypic assessment, we found that adults exposed to thermal fluctuations tolerated high temperatures better than those at constant temperatures irrespective of whether the flies were sampled at the high or low temperature point of a fluctuation cycle. Thus, fluctuations resulted in a constitutive improvement in heat tolerance, as also shown by Manenti et al. (2018). Larval high temperature tolerance was much higher than that of the adult flies (Krebs, 1999). Moreover, developmental acclimation to fluctuating temperatures increased larval heat tolerance during the day (high temperature time point), while differences in larval tolerance at night (low temperature time point) did not differ significantly (Figure 2). This could reflect different adaptive avenues among life stages, where larval stages (being less mobile) rely on physiological tolerance whereas adult flies could more efficiently rely on behavioural avoidance of stressful temperatures (Feder et al., 1997). A higher heat tolerance for the immobile pupal stage compared to adults is commonly found (e.g., Enríquez &
4.1 Sex- and life stage-specific differences in gene expression at fluctuating temperatures

Using the genes identified to be differentially expressed between constant and fluctuating regimes (overall analyses) within larvae, adult males and adult females, we intersected the three groups to obtain genes that were commonly and uniquely expressed by each group (Figure S2). GO analyses of these genes revealed that neurogenesis was differentially affected by fluctuations in males when compared to either larvae or females. Processes relating to mitochondrial translation were also enriched in males relative to larvae (Table 4). Larval responses to thermal fluctuations were mainly driven by the metabolism of xenobiotics and drug metabolism, both mediated by cytochrome P450 (Table 4). In contrast, females responded uniquely to fluctuating temperatures by negatively regulating histone modifications and regulating processes relating to phototransduction (Table 4). Negative regulation of histone modifications was also seen in our analyses using masigpro wherein female gene expression at the fluctuating regime tracked the fluctuations in temperature (pattern E, Table 2). Whether this enables better acclimation responses to thermal fluctuations should be further investigated in future studies.

Colinet, 2017), supporting the notion of generally improved heat tolerance for the less mobile life stages. Thus, fluctuating temperatures generally induced beneficial acclimation in heat tolerance, although differently in the two life stages.

FIGURE 6 Differentially expressed genes identified by masigpro and clustered into nine expression patterns for male samples based on k-means clustering. The x-axis corresponds to the time point in days (High2 and High3 correspond to the high temperature point, and Low2 and Low3 to the low temperature point of the 7 and 13 fluctuation, respectively) and y-axis to the expression value. For plotting, the time points are considered as factorial
| Pattern | Larvae | | Male |
|---------|--------|-----------------|--------|
| **Pattern A** | **KEGG** | dme04141: Protein processing in endoplasmic reticulum | dme032504: multicellular organism reproduction |
| | **BP** | dme04144: Endocytosis | GO:0007610: behaviour |
| | | GO:0032504: multicellular organism reproduction | GO:0032504: multicellular organism reproduction |
| | | GO:0035080: heat shock-mediated polytene chromosome puffing | GO:0006986: response to unfolded protein |
| | | GO:0009408: response to heat | GO:0001666: response to hypoxia |
| | | GO:0006986: response to unfolded protein | GO:0045297: post-mating behaviour |
| **Pattern B** | **KEGG** | n/a | n/a |
| **Pattern C** | **KEGG** | n/a | n/a |
| **Pattern D** | **KEGG** | n/a | n/a |
| **Pattern E** | **KEGG** | n/a | n/a |
| **Pattern F** | **KEGG** | n/a | n/a |

(Continues)
| Male | | |
|---|---|---|
| Pattern G | dme01200: Carbon metabolism | |
| KEGG | dme00020: Citrate cycle (TCA cycle) | |
| | dme01130: Biosynthesis of antibiotics | |
| | dme01230: Biosynthesis of amino acids | |
| | dme01100: Metabolic pathways | |
| BP | GO:0006099: tricarboxylic acid cycle | |
| Pattern H | n/a | |
| KEGG | n/a | |
| BP | GO:0032504: multicellular organism reproduction | |
| Pattern I | n/a | |
| KEGG | n/a | |
| BP | GO:0000209: protein polyubiquitination | |
| Female | | |
| Pattern A | dme00020: Citrate cycle (TCA cycle) | |
| KEGG | dme01200: Carbon metabolism | |
| | dme01130: Biosynthesis of antibiotics | |
| | GO:0006099: tricarboxylic acid cycle | |
| BP | n/a | |
| Pattern B | dme00190: Oxidative phosphorylation | |
| KEGG | dme01100: Metabolic pathways | |
| | dme00020: Citrate cycle (TCA cycle) | |
| BP | GO:0030239: myofibril assembly | |
| | GO:0006120: mitochondrial electron transport, NADH to ubiquinone | |
| | GO:0015992: proton transport | |
| | GO:0045214: sarcomere organization | |
| Pattern C | n/a | |
| KEGG | n/a | |
| BP | GO:0022008: neurogenesis | |
| Pattern D | n/a | |
| KEGG | n/a | |
| BP | n/a | |
| Pattern E | n/a | |
| KEGG | n/a | |
| BP | GO:0031057: negative regulation of histone modification | |
| Pattern F | n/a | |
| KEGG | n/a | |
| BP | GO:0001731: formation of translation preinitiation complex | |
| Pattern G | n/a | |
| KEGG | n/a | |
| BP | n/a | |
| Pattern H | dme00190: Oxidative phosphorylation | |
| KEGG | dme01100: Metabolic pathways | |
4.2 | Thermal fluctuations activate temperature stress responses

For both larvae and adults, many genes typically expressed in response to stressful high temperature were also enriched at fluctuating temperatures (Overall, Table 3), including differential regulation of the GO category “response to heat,” which included genes for major heat shock proteins (Hsp83, Hsp70Bb, Hsp70Ba). Hsp70 and Hsp83, typically induced by heat, are also induced by cold and play an important role in repairing chill injuries in Drosophila melanogaster in response to thermal fluctuations (Colinet et al., 2010). Hsp70 is also known to modulate glutathione-related enzymes in canine kidney cells and this could explain the co-expression of genes for “Glutathione-s-transferase” along with the heat shock proteins, thereby mitigating oxidative stress (Guo et al., 2007). The maximum temperature reached during fluctuations in this study (27°C) is not expected to elicit a strong induction of the heat stress response, but several studies point to a fine-tuned regulation and adaptive role of Hsps under more benign thermal conditions (Sørensen et al., 2003, 2017). The current study supports a similar role of the stress response under repeated fluctuating conditions. GO analysis of the female transcriptome across time points (Overall, Table 3) revealed enrichment of genes belonging to ion transmembrane transport (Ca^{2+} and K^{+}). Similar GO categories are known to be enriched for low temperature acclimation in Drosophila but have not previously been found at fluctuating temperatures (Chen et al., 2015a; Enríquez & Colinet, 2019). However, given that the temperature in our fluctuating regime reaches a minimum of 11°C—in contrast to the constant 13°C used by Chen et al. (2015a) for their experiment—we hypothesize such a response could be elicited by temperature fluctuations. These categories are implicated in ion homeostasis and could prevent or protect against chill injury during the low temperature phase of a fluctuation (Torson et al., 2015). Several genes involved in the oxidative phosphorylation pathway and mitochondrial electron transport system were also differentially regulated in the females (female patterns B and H, Figure 5, Table 2). Temperature dependence of oxidative phosphorylation has been established in endothermic and ectothermic species (Lemieux et al., 2017; Sacktor & Sanborn, 1956), where it is essential for energy production via ATP generation (Tripoli et al., 2006). This could constitute a physiological cost for continuous acclimation to fluctuating temperatures. Moreover, up-regulation of genes involved in this pathway counteracts the inhibition of ATP generation at low temperatures (Kerbler et al., 2019), and thus acclimation to fluctuating temperatures could allow for optimal performance under ecologically relevant temperature conditions. It is not clear from this study exactly which part of the fluctuation induces each response. Further studies are needed to discern responses to the changing temperature from the responses to reaching the high and low temperature points, respectively. Regardless, these two are integral parts of variable temperature responses to which the identified processes are associated.

4.3 | Preventing oxidative stress is a priority for larvae in fluctuating temperatures

For larvae, KEGG and GO analysis of the differentially expressed genes across time points (from the “overall” analyses) showed important roles for genes critical for preventing oxidative stress. It is likely that the low temperatures experienced during thermal fluctuations result in chill injury by compromising the antioxidant system, while the higher temperatures experienced could counteract these effects by allowing for repair (Joanisse & Storey, 1996; Kostál et al., 2007; Lalouette et al., 2011). Important pathways belonging to the “Cytochrome P450” superfamily, “Glutathione metabolism” and GO categories “Glutathione peroxidase activity” and “Glutathione transferase activity” were enriched among the genes differentially up-regulated (Table 3), which was also reflected in larval gene expression pattern D (Figure 4, Table 2). These processes are central to coping with stressful conditions in D. melanogaster (Bourg, 2001).

4.4 | Transcriptional and post-transcriptional modulations impact acclimation responses in males

GO analysis of the male transcriptome across time points (from the “overall” analyses) revealed several categories involved in transcription to be enriched in the genes differentially up-regulated by fluctuations. Among the genes differentially down-regulated by fluctuations, we saw a similar pattern with GO categories such as “rRNA processing,” “maturation of LSU-rRNA,” “maturation of SSU-rRNA” and “ribosome biogenesis” being enriched (Table 3).
### TABLE 3  KEGG and GO analysis of the overall regime effect across time points obtained through "overall" analyses

|               | Larvae                                                                 | Female                                                                 |
|---------------|------------------------------------------------------------------------|------------------------------------------------------------------------|
| **KEGG**      | dme00982: Drug metabolism – cytochrome P450                           | n/a                                                                   |
|               | dme00980: Metabolism of xenobiotics by cytochrome P450                 |                                                                        |
|               | dme00480: Glutathione metabolism                                       |                                                                        |
| **BP**        |                                                                        |                                                                        |
| Up            | GO:0006749–glutathione metabolic process                              | GO:0002181–cytoplasmic translation                                      |
| BP            |                                                                        |                                                                        |
| KEGG          | dme00982: Drug metabolism - cytochrome P450                           |                                                                        |
|               | dme00980: Metabolism of xenobiotics by cytochrome P450                 |                                                                        |
|               | dme00480: Glutathione metabolism,                                     |                                                                        |
|               | dme04141: Protein processing in endoplasmic reticulum                 |                                                                        |
| **BP**        | GO:0035080–heat shock-mediated polytene chromosome puffing            | GO:0002181–cytoplasmic translation                                      |
| Down          |                                                                        |                                                                        |
| **KEGG**      |                                                                        |                                                                        |
|               |                                                                        |                                                                        |
| **BP**        |                                                                        |                                                                        |
| Up            | GO:0022008–neurogenesis                                               |                                                                        |
| Down          |                                                                        |                                                                        |
| **KEGG**      |                                                                        |                                                                        |
|               |                                                                        |                                                                        |
| **BP**        |                                                                        |                                                                        |
| Up            | GO:0006351–rRNA processing                                            |                                                                        |
|               | GO:0006364–rRNA processing                                            |                                                                        |
|               | GO:000462–maturation of SSU-rRNA from tricistronic rRNA transcript    |                                                                        |
|               | (SSU-rRNA, 5.8S rRNA, LSU-rRNA)                                        |                                                                        |
|               | GO:0099267–cellular response to starvation                             |                                                                        |
|               | GO:0042254–ribosome biogenesis                                         |                                                                        |
|               | GO:000470–maturation of LSU-rRNA                                       |                                                                        |
GO terms "translation," "centrosome organization," and "ribosomal small subunit assembly" were constitutively up-regulated in the FR male transcriptome relative to CR (male pattern C, Figure 6, Table 2). Response to temperature is often mediated by transcriptional and post-transcriptional modulations in a variety of species including plants and animals (Gotic & Schibler, 2017; Guerra et al., 2015). Our results suggest that the transcriptional machinery is greatly affected by temperature fluctuations and that post-transcriptional modulations are crucial for responding to thermal variabilities. Why this was particularly prominent in male flies is presently unclear.

4.5 | Canalization of gene expression facilitates the response to thermal variability in females

Our transcriptomic analysis using MA SigPro revealed signatures of canalization of gene expression in the adult female flies. To our surprise, several patterns identified by k-means clustering had genes with stabilizing expression in the fluctuating regime compared to the constant temperature regime (Figure 5). This was counterintuitive as we would have expected fluctuations to induce altered gene expression in line with the temperature change. We have no clear explanation for this sex-specific pattern, which was observed in a substantial proportion of the identified genes. Nevertheless, canalization of gene expression has been proposed as one possible mechanism enabling adaptation to low temperatures (increased cold tolerance) in temperate D. melanogaster (von Heckel et al., 2016). If such patterns of canalization also provide enhanced heat tolerance in a fluctuating environment, the realized benefits of such a mechanism in the wild—where temperatures fluctuate on a spatiotemporal scale—could be substantial and would constitute a novel class of temperature response.

4.6 | Phototransduction is crucial for responding to thermal variability

Drosophilids sense temperature through sensory channels such as the Transient Receptor Potential (TRP) channels (reviewed in Table 3 (Continued)

| Female |
|--------|
| Up |
| KEGG |
| BP |
| GO:0057005: Phototransduction – fly |
| GO:0055085: transmembrane transport |
| GO:0007601: visual perception |
| GO:0072499: photoreceptor cell axon guidance |
| GO:0007186: G-protein coupled receptor signalling pathway |
| GO:0006813: potassium ion transport |
| GO:0007424: open tracheal system development |
| GO:0070588: calcium ion transmembrane transport |
| GO:0055114: oxidation-reduction process |
| GO:0046845: branched duct epithelial cell fate determination, open tracheal system |
| GO:0007498: mesoderm development |
| GO:0007156: homophilic cell adhesion via plasma membrane adhesion molecules |

| Down |
| BP |
| GO:0007605: sensory perception of sound |
| GO:0032504: multicellular organism reproduction |
| GO:0031057: negative regulation of histone modification |
| GO:0006952: defense response |
| GO:0050830: defense response to Gram-positive bacterium |
| GO:0005615: extracellular space |
| GO:0008235: metalloexopeptidase activity |
| GO:0030145: manganese ion binding |
| GO:0004177: aminopeptidase activity |

| Common |
| KEGG |
| BP |
| GO:0004141: Protein processing in endoplasmic reticulum |
| GO:0009408: response to heat |
| GO:0006457: protein folding |

Note: All terms having an adjusted \( p < .1 \) are reported.
Abbreviations: BP, biological processes; KEGG, Kyoto Encyclopedia of Genes and Genomes; n/a, not applicable.
TABLE 4  KEGG and GO analysis for the genes identified in the “overall” analysis and differentially regulated between male, female and larval groups at an adjusted p < .01 (Figure S2)

Differentially expressed genes common between larvae, males and females

| KEGG          | GO:0022008–neurogenesis |
|---------------|-------------------------|
| BP            | GO:0032543–mitochondrial translation |
|               | GO:006351–transcription, DNA-templated |
|               | GO:000381–regulation of alternative mRNA splicing, via spliceosome |
|               | GO:007476–imaginal disc-derived wing morphogenesis |
|               | GO:0042254–ribosome biogenesis |
|               | GO:006357–regulation of transcription from RNA polymerase II promoter |

Note: All terms having an adjusted p < .1 are reported. Abbreviations: BP, biological processes; KEGG, Kyoto Encyclopedia of Genes and Genomes; n/a, not applicable.

Barbagallo & Garrity, 2015). These also form part of the Drosophila phototransduction pathway, which has been increasingly implicated in thermosensation (Barbagallo & Garrity, 2015; Nielsen et al., 2006). Sørensen, Schou, et al. (2016) also proposed a novel role for phototransduction in regulating thermal acclimation to fluctuating temperatures. We reveal a similar role for the environment-sensing phototransduction pathway in the female fruit fly. Many genes involved in phototransduction were differentially up-regulated in the female flies exposed to temperature fluctuations (Table 3). Moreover, differential up-regulation of Ca²⁺ channels in our study (Table 3) also supports the role for phototransduction in mediating acclimation responses to thermal fluctuations as Ca²⁺ channels are essential for mediating TRP activity and consequently the phototransduction pathway (Hardie & Juusola, 2015). Although previously implicated (Sørensen, Schou, et al., 2016), we did not find evidence for phototransduction-mediated thermal acclimation in male flies in this study. Nor did we observe such a mechanism in the larval stages.

5 | CONCLUSIONS

Consideration of ecologically relevant thermal fluctuations is essential for estimating thermal tolerances and predicting acclimation potential to climatic variability. Even more important is to understand the molecular mechanisms driving acclimation responses to varying environmental conditions, especially since thermal fluctuations can accelerate the evolution of thermal tolerance (Schaum et al., 2018). Here we show that acclimation to fluctuating temperatures constitutively improves heat tolerance across the adult life stage in D. melanogaster, while larval tolerance is affected in a temporal manner. We further show that the molecular basis of phenotypic plasticity is intrinsically linked to the life stages and the sex of the adult flies. Several mechanisms were shared between all experimental animals, whereas some were uniquely activated based on life stage/sex. These were regulated in a temporal manner with mechanisms also being contributed from the low and high temperature phases of a thermal fluctuation. We propose canalization of gene expression, and phototransduction as crucial mechanisms that allow female flies to respond to thermal variability via environmentally induced phenotypic plasticity.
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CONFLICT OF INTERESTS
The authors declare that we have no conflicts of interest.

AUTHOR CONTRIBUTIONS
P.V.S. and J.G.S. conceived the idea and designed the study, P.V.S. collected the data, analysed the data and wrote the original draft. J.G.S. edited and reviewed the draft. Both authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The gene expression data discussed in this paper have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession no. GSE150450 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150450). All phenotypic data have been deposited in the Dryad digital data repository and can be accessed at https://doi.org/10.5061/dryad.2v6wwpzjh.

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