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Expression of thermal tolerance genes in two *Drosophila* species with different acclimation capacities

Jesper Givskov Sørensen[a,*], Marta Puig Giribets[a,b], Rosa Tarrío[b], Francisco Rodríguez-Trelles[b], Mads Fristrup Schou[a,c], Volker Loeschcke[a]

[a] Department of Bioscience, Genetics, Ecology and Evolution, Aarhus University, Ny Munkegade 116, 8000, Aarhus C, Denmark
[b] Department de Genètica i de Microbiologia, Grup de Genòmica, Bioinformàtica i Biologia Evolutiva (GGBE), Universitat Autonòma de Barcelona, 08193, Bellaterra, Barcelona, Spain
[c] Department of Biology, Lund University, Lund, SE-22362, Sweden

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ABSTRACT

Heat tolerance increases at higher acclimation temperatures in *D. melanogaster*, but not in *D. subobscura*. The two species represent separate lineages of the subgenus Sophophora of *Drosophila* with contrasting tropical African and temperate Palearctic evolutionary histories. *D. melanogaster* has five copies of the inducible *hsp70* gene distributed in two clusters, named A (with two copies) and B (three copies), while *D. subobscura* has only two copies arranged similarly to cluster A of *D. melanogaster*. The *hsp70s* of the two species also differ in their cis-regulatory regions, with *D. melanogaster* exhibiting features of a faster and more productive promoter. We predicted that the interspecific variation in acclimation capacity of heat tolerance is explained by evolved variation in expression of the major group of heat shock proteins. To test this prediction, we compared basal levels of gene expression at different developmental temperatures within each of the two species. Furthermore, we explored the heat hardening dynamics by measuring the induction of gene expression during a ramping assay. The prediction of a stronger heat shock protein response in *D. melanogaster* as compared to *D. subobscura* was confirmed for both long-term acclimation and short-term hardening. For *D. melanogaster* the upregulation with temperature ramping ranged from less than two fold (*hsp26*) to 2500 fold (*hsp70A*) increase. In all cases induction in *D. melanogaster* exceeded that of *D. subobscura* homologs. These differences correlate with structural differences in the regulatory regions of *hsp70,* and might explain differences in acclimation capacity among species. Finally, in *D. melanogaster* we found an indication of an inverse relationship between basal and induced levels of *hsp70A* and *hsp83* expression, suggesting a divergent role for thermal adaptation of these genes at benign and stressful temperatures, respectively.

1. Introduction

Small ectotherms typically have body temperatures that are assumed to quickly equilibrate with the prevailing temperatures (Angilletta et al., 2010; Frazier et al., 2006). As temperatures fluctuate markedly across short spatial and temporal scales in nature, species are expected to benefit from the ability to express phenotypic plasticity in thermal tolerance traits (thermal acclimation) (Angilletta, 2009), in which exposure to high temperatures in the short-term (hardening) or long-term (acclimation) leads to increased heat tolerance (Hoffmann, 1995; Lindquist, 1986).

The mechanisms involved in acclimation responses are not fully understood. Most well established is the so-called heat stress response, induced by exposure to acute heat stress, which involves expression of molecular chaperones (e.g. heat shock proteins) (Feder and Hofmann, 1999). The heat shock proteins are highly conserved among species and consist of several families, serving different specialized functions. These functions are all related to maintenance and/or re-establishment of cellular functions (e.g. re-folding or degradation of denatured proteins) under normal conditions and under stress (Feder and Hofmann, 1999; Sørensen et al., 2003). Within the major heat shock protein family, Hsp70, at least ten paralogs originating from several duplication events exist. These can be divided into several cognates, i.e. constitutively expressed heat shock cognates (*hsc70*), and several purely inducible *hsp70* gene copies. In addition to Hsp70 other main heat shock protein families in *Drosophila* include Hsp90 (constitutive and further

* Corresponding author.
E-mail address: jesper.soerensen@bios.au.dk (J.G. Sørensen).

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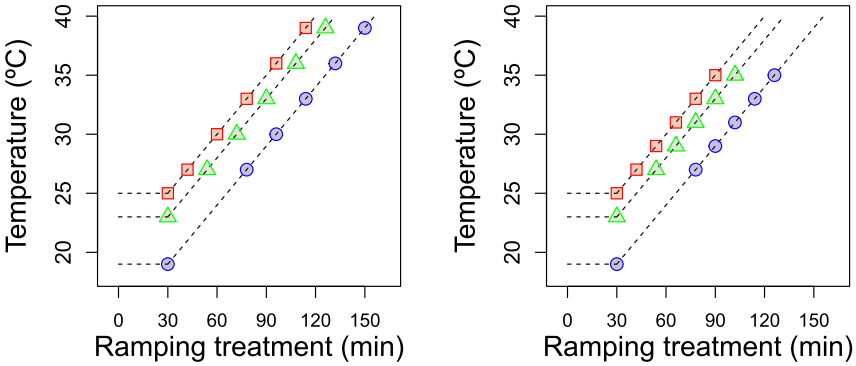

Fig. 1. Experimental design: *D. melanogaster* (left) and *D. subobscura* (right) developed from egg to adults at constant 19, 23 or 25 °C. Adult males were thereafter transferred to a temperature-controlled water bath. Samples were taken and snap-frozen after 30 min equilibrium at the developmental acclimation temperature and then at different predefined temperatures during a ramping assay at a rate of 0.1 °C/min. The samples were investigated for gene expression of heat shock proteins using qPCR.

inducible), Hsp60 (the main mitochondrial heat shock protein) and a range of small heat shock proteins (sHsps; Hsp22, Hsp23, Hsp26, Hsp27) (Feder and Hofmann, 1999; Sørensen et al., 2003). The heat stress response is transient and, with some delay, expression levels return to normal after the stress has ended (Sørensen et al., 2005a). Studies of mutant lines of e.g. *Drosophila* and yeast document the crucial importance of the heat shock proteins for survival to high temperature stress (Feder et al., 1996; Parsell and Lindquist, 1994). However, evolutionary adaptation (both natural and laboratory) to high temperatures in *Drosophila* seems to lead to a reduced sensitivity of the heat stress response as a consequence of the higher baseline tolerance (Sørensen et al., 2003). Thus, the level of induction of the heat stress response (and the associated heat shock proteins) seems to reflect the requirement for damage repair and prevention of further damage, at least within a species (Ananthan et al., 1986).

Their massive induction upon heat shock makes heat shock proteins prime candidates for adaptation to high temperatures (Feder and Hofmann, 1999). High levels of conservation in protein coding sequence typical of heat shock genes attests for strong negative selection, thereby evolutionary adaptation to heat stress should occur mainly through regulatory noncoding changes affecting gene expression (Sørensen et al., 2003). Studies on species of *Drosophila* and many other organisms suggest that heat tolerance is a complex trait, likely affected by several mechanisms. Thus, while heat shock proteins are crucial for survival to acute heat shock, selection for increased heat tolerance leads to a reduced expression of Hsp70 in response to a high temperature shock (Nielsen et al., 2005; Sørensen et al., 2003). Furthermore, while *hsp* transcripts are induced to high abundance during a thermal ramping, the critical thermal maximum is only marginally increased (Sørensen et al., 2013) and no global transcriptomic signature of heat shock proteins is detected for developmental acclimation to different temperatures (Sørensen et al., 2016b). Finally, while clinal patterns in heat tolerance and Hsp70 expression levels have been found among geographical populations (Sørensen et al., 2005b), no clear correlation among the same traits was found for within population variation in adult *Drosophila* (Jensen et al., 2009). Thus, the role of heat shock protein regulation in thermal acclimation and heat hardening during temperature ramping remains unresolved.

In *Drosophila*, developmental acclimation generally leads to a beneficial effect on heat tolerance, with an improvement of the critical thermal maximum (CTmax) of ca. 0.1 °C per °C increased acclimation temperature (Schou et al., 2017b; Sørensen et al., 2016a). However, variation among species includes non-significant or even negative acclimation responses. For example, in the study by Schou et al., (2017b) the temperate species *D. subobscura* showed a decrease in CTmax with higher developmental acclimation temperatures. Furthermore, an investigation of several aquatic dipterans suggests that the typical characteristics of the heat stress response of *Drosophila* species might not constitute the general pattern of insects and that constitutive levels of Hsp70 expression play a prominent role for evolutionary adaptation (Zatsepina et al., 2016). As the majority of studies have concentrated on *D. melanogaster*, interspecific contrasts are needed to elucidate the generality of the current knowledge.

D. melanogaster and *D. subobscura* are two *Drosophila* representatives of tropical African and temperate Palearctic origins, respectively (Throckmorton, 1975) and differ in genetic architecture of the major inducible heat shock protein genes (*hsp70*). *D. melanogaster* has five copies of this inducible gene distributed in two cytologically distinct clusters (clusters A with two copies and B with three copies), while *D. subobscura* has only two copies of the inducible *hsp70*. The two copies in *D. subobscura* are arranged as in cluster A of *D. melanogaster*: closely spaced head-to-head, which is thought to be the ancestral configuration in dipterans (Bettencourt and Feder, 2001). Based on these differences in acclimation capacity and genetic architecture in candidate genes, we predict that interspecific variation in acclimation and hardening capacity is explained by variation in expression of the major groups of heat shock proteins. In addition to the major inducible *hsp70*, we investigated representatives of heat shock cognates (*hsc70-4*) and small heat shock proteins (*hsp23* and *hsp26*) in both species.

Here we test this prediction by comparing basal and induced heat shock gene expression levels between *D. subobscura* and *D. melanogaster*. We delineated the induction profiles for heat shock genes in each of three developmental temperatures using the values obtained at six different time points during a thermal ramping assay (see Fig. 1). With these data we tested the following hypotheses: I) Within species, developmental acclimation affects the basal expression levels of heat shock proteins and thus explains developmental acclimation effects on CTmax (as a measure of heat tolerance). II) Within species, developmental acclimation affects the induction dynamics (during a ramping assay) and thus explains developmental acclimation effects on CTmax. III) Differences between species in either of the above, i.e. basal (as induced by developmental acclimation) or induced (as induced by thermal ramping) expression levels could explain the difference in basal CTmax and/or the difference in acclimation ability.

2. Materials and methods

2.1. Fly maintenance and acclimation

We used populations of *D. melanogaster* and *D. subobscura* (established from twenty-five wild caught female flies, collected in Denmark in 2013 and 2014, respectively; Schou et al., 2017b). Flies were reared on standard oatmeal–sugar–yeast–agar *Drosophila* medium at 19 °C and a 12/12 h light/dark cycle. Since collection it is likely that some laboratory adaptation has occurred, but heat tolerance is expected to be unaffected and species differences maintained (Krebs and Loeschcke, 1997; Maclean et al., 2018). Experimental flies were density controlled to reduce variation among individuals and to synchronize emergence. For *D. melanogaster*, batches of ~20 two-day old flies were transferred to empty shell vials containing a spoon filled with standard *Drosophila* medium and stored at 19 °C for egg laying. In *D. subobscura*, numerous (> 100) 7–14 days old adults were placed in bottles with fresh medium

enriched with yeast to promote egg laying and stored at 19 °C. Eggs were collected from the medium and transferred in batches of 40 (± 3) eggs to 7 mL food vials. For each species, vials with eggs were randomly distributed in similar proportions across temperature-controlled chambers for the three temperature regimes (constant 19 °C, 23 °C and 25 °C, respectively) where they stayed until adults emerged. For each temperature male flies (sexed after being anaesthetized with CO_2) were collected within 24 h of emergence. Flies were distributed in groups of five individuals in fresh food vials and replaced at their respectively temperature regime. In total 75 vials for each species and 25 for every temperature regime were collected. We only investigated males to avoid the potential variability associated with female reproductive status, however, we expect the general acclimation response to be similar between sexes (Schou et al., 2017a).

23.2. Ramping assay

Males from each thermal regime and species were exposed to a ramping assay where flies were sampled for candidate gene expression at six different pre-set temperature points. The males were 5–6 days old and transferred to individual 5 mL plastic tubes using an aspirator without removing them from their thermal regime. Prior to the ramping, the tubes (n = 24 with five flies each) were transferred to a preheated, temperature-controlled water bath in dim light (Fig. 1) corresponding to the temperature regime of origin (19°C, 23 °C or 25 °C). After 30 min at this constant temperature, the first sampling (T_0) took place by removing four replicates and covering them with dry ice to kill the flies and prevent RNA degradation. Hereafter a ramping of the temperature with a constant rate of $\Delta T = 0.1$ °C/min was initiated. Subsequently, the remaining five sets of samples were removed and covered by dry ice at the five remaining predetermined temperature points that were common to the three thermal regimes but differed between the two species (27°C, 29 °C, 31 °C, 33 °C, 35 °C for *D. subobscura* and 27°C, 30°C, 33°C, 36°C, 39°C for *D. melanogaster*). The reasons for having two different temperature sets were i) *D. subobscura* flies are more sensitive to high temperatures than their *D. melanogaster* counterparts and ii) maximum protein (Hsp70) or RNA (*hsp70*) abundance is attained at about 37 °C in *D. melanogaster* (Krebs, 1999) but at only 32 °C in *D. subobscura* (Calabria et al., 2012), respectively. After finishing the ramping assay, samples were stored at −80 °C until analysis. As the different developmental acclimation regimes cause different developmental times, it was necessary to expose each species and treatment to individual ramping assays to avoid large age differences of the exposed flies. A previous study has documented high repeatability and low variation in results among assays using the same protocols and equipment (Schou et al., 2017b).

2.3. Gene selection and primer design

The *D. melanogaster* genes selected for quantification of expression have previously been described as stress-responsive (Bettencourt et al., 2008), and include representatives of the small HSPs (*hsp23*, *hsp26*, *hsp27*), two conserved paralogs of the Hsp70 protein family (*hsp70A*, *hsp70B* clusters), the Hsp70 protein cognate 4 (*hsc70-4*), the mitochondrial Hsp60 protein (*hsp60*), and Hsp83 (*hsp83;* the *Drosophila* homolog of mammalian HSP90). Primers were designed to amplify a relatively short (~100 bp) conserved fragment in the coding region of each gene, and sequences for primer design were retrieved from the *D. melanogaster* genome using the NCBI (https://www.ncbi.nlm.nih.gov/) or FlyBase genbanks (http://flybase.org/). To identify orthologs of the same loci in *D. subobscura*, the sequences of three *D. melanogaster* genes (*hsp23*, *hsp26* and *hsc70-4*) were used to conduct BLASTN searches against a *de novo* genome-draft assembly of our laboratory stock of the *ch-cu* strain (Rosa Tarrío and Francisco Rodríguez-Trelles; unpublished results). The assembly was generated upon request by Macrogen Inc (Seoul, South Korea) using 54 924 584 300 bp long mate-paired reads from a Miseq run of a 10 kb insert library (referred to as 2 × 300 MP10 assembly). In the case of *hsp70*, qPCR primers were designed using published sequence information (Puig Giribets et al., 2019). After retrieval of positive contigs, primers were designed for the three *D. subobscura* genes to amplify a fragment of similar length that overlapped as much as possible with its homologous counterpart in *D. melanogaster*. Taking into account their high degree of conservation at the sequence level (Puig Giribets et al., 2019), a single primer pair was used to amplify the same region in the two *hsp70* duplicates of *D. subobscura*. Oligonucleotides for PCR amplification and sequencing were designed using the online tool Primer3Plus (https://primer3plus.com/; Untergasser et al., 2007). Parameters for primer design were the following: Melting temperature (Tm°): 59 °C – 61 °C. Length: 20–22 bp. Amplicon length: 80–120 bp. %GC: 45%–55%. Accession numbers and primer sequences for the investigated genes in the two species are listed in Supplementary Table S1.

2.4. Gene expression

To quantify expression levels, RNA was extracted from the four replicates per treatment and species using an Omega MicroElute Total RNA Kit (VWR, Søborg, Denmark) according to the manufacturer's instructions. RNA concentration per sample was determined spectrophotometrically using a Qubit 2.0 fluorometer (Invitrogen, Nærum, Denmark), converted to cDNA using the Omniscript Reverse Transcriptase kit (Qiagen, Vedbæk, Denmark) and an Anchored Oligo (dT)20 primer (Invitrogen), and diluted to a final concentration of 3 ng/µl total RNA. Quantitative PCR (qPCR) assays for the target genes were run on a Stratagene MxPro - MX3005P qPCR system (AH Diagnostics, Aarhus, Denmark) as described in Waagner et al. (2013). Raw qPCR data was quality controlled by inspection of PCR and melting curvature (to ensure that a single product was amplified) and analyzed with Data Analysis for Real-Time PCR Version 1.0; Peirson et al. (2003) and normalized for each species using the NORMA-Gene algorithm (Heckmann et al., 2011) as described in Waagner et al. (2013).

2.5. Analyses

The gene expression data were separated in two subsets. The first subset contained the basal gene expression at the respective developmental acclimation temperatures (19, 23 or 25 °C). The second subset contained the gene expression data obtained from flies exposed to ramping temperatures (≥ 27 °C). For both subsets log-transformation of the gene expression level was necessary as non-transformed data violated the assumption of homogeneity of variance. In the first subset, changes in basal expression levels with increasing developmental acclimation temperatures were investigated using gene-specific linear models containing expression as a response variable and developmental acclimation temperature as a continuous predictor variable. In the second subset, the change in expression with increasing temperature during a thermal ramping was investigated by constructing full models for each gene containing the continuous predictor variables developmental acclimation temperature and ramping temperature at sampling. The full models also considered the interaction between these two main factors. To account for observed curvature in the reaction norm of expression level across ramping temperatures, we modelled ramping temperature as a quadratic effect. Both acclimation and ramping temperatures were z-transformed (centered and scaled) to generate biologically-meaningful intercept estimates. Before testing the impact of individual terms in the models, we compared the full model to a null model to verify that the main factors and their interaction explained a significant part of the variance in the data set. Upon significance, we performed sequential model reduction and model comparisons with F-tests to find the minimal adequate model (Crawley, 2013). In the case of a significant interaction or main factor, model reduction was stopped for the involved predictor variables. All statistical analyses were

Table 1
Results from the linear models for effect of developmental acclimation temperature and effects of ramping induced gene expression of eight candidate genes in *D. melanogaster*. The effect of ramping was evaluated by both a linear and a 2nd order polynomial component. See Tables S2 and S3 for the results of the full and final models after reduction. *P < 0.05; **P < 0.01; ***P < 0.001; ns not significant; - signifies that no test of significance was performed either because the full model was non-significant or because a higher order factor was significant.

	hsp23	*hsp70A*	*hsp26*	*hsc70-4*	*hsp60*	*hsp27*	*hsp70B*	*hsp83*
Ramping induced gene expression								
Ramping (linear/2nd order poly)	(***/ns)	(−/−)	(-/**)	–	–	(***/ns)	(-/***)	(-/***)
Developmental acclimation	ns	–	ns	–	–	ns	ns	*
Ramping x Dev. accl	ns	***	ns	–	–	ns	ns	ns

completed in R (http://www.R-project.org).

3. Results

3.1. Basal expression levels (different developmental temperatures)

Our qPCR probes did not allow us to distinguish between genes from the same cluster of the major heat shock protein Hsp70, so for simplicity we will from here on refer to those genes indistinctly either as genes or clusters *hsp70A* and *hsp70B*. For *D. melanogaster* two genes (*hsp70A* and *hsp70B*) showed a significant increase in basal expression level with increasing developmental acclimation temperature (Table 1, Table S2). In both cases the induction was modest, with expression levels around 2.5 fold higher at 25 °C as compared to 19 °C (Fig. 2, Fig. S1). Two other genes showed a similar tendency (*hsp23* and *hsc70-4*), although not significantly so (Figs. 2 and 3, Table S2). For *D. subobscura* none of the investigated transcripts showed a significant increase in basal gene expression level with developmental acclimation temperature (Figs. 2 and 3, Table S4).

3.2. Ramping induced expression

For *D. melanogaster* we found quite diverse responses to ramping among the eight genes investigated (Figs. 2 and 3, Table 1, Fig. S1, Table S3). Two genes (*hsp60* and *hsc70-4*) showed no significant change in expression levels in response to temperature ramping, although *hsc70-4* was marginally significant. The remaining six genes all showed upregulation with temperature ramping with fold changes ranging from less than two fold increase (*hsp26*) to a 2500 fold increase (*hsp70A*). Statistical analyses showed that the relationship between ramping temperature and expression level was of a linear nature (Table 1, Table S3). For *hsp23* and *hsp27* only ramping temperature had a significant linear effect on gene expression, with no significant effect of developmental temperature or the interaction between ramping and developmental temperature. For *hsp26* and *hsp70B* gene expression was non-linearly (through a significant quadratic component) related to ramping temperature. No significant effect of developmental temperature or the interaction was found. For two genes (*hsp70A* and *hsp83*) acclimation temperature significantly explained variation in expression during ramping. For *hsp83* a negative model estimate suggests that increasing developmental acclimation temperature led to a reduced gene expression during thermal ramping. The same was observed for *hsp70A*, but here the interaction between ramping temperature and developmental acclimation temperature was significant. Investigation of the model predictions showed that this interaction was driven by flies acclimated at low temperatures having an increased gene expression at lower ramping temperatures but not at high ramping temperatures. Thus, at 19 °C developmental acclimation the expression levels were increased (or decreased at 25 °C acclimation) at the lower ramping temperatures, but this difference disappeared at higher ramping temperatures. This could indicate that lower developmental acclimation made flies more sensitive early on during the ramping.

For *D. subobscura* we generally found more modest responses in the expression to thermal ramping as compared to *D. melanogaster* (Figs. 2 and 3). The treatments had no effect on the expression of *hsp26* and *hsc70-4*. The two other genes (*hsp70*, i.e., the counterpart of the *D. melanogaster hsp70A* cluster, and *hsp23*) showed upregulation with increasing temperature during ramping, with fold changes not exceeding 100 fold. For both of these genes a significant linear relationship between expression level and ramping temperature was found (Table 2, Table S5).

4. Discussion

Due to the massive induction in response to heat shock, heat shock proteins are prime candidates for adaptation to high temperatures (Feder and Hofmann, 1999). The high interspecific similarity in amino acid sequences between genes coding for the heat shock response suggests a high degree of functional constraint leaving adaptive evolution to target the regulation of expression of the heat shock genes (Sørensen et al., 2003). However, the role of regulation of heat shock proteins for thermal acclimation and that of heat hardening during heat tolerance (ramping) assays remain unresolved.

D. melanogaster and *D. subobscura* show different developmental acclimation responses to increasing temperature. While *D. melanogaster* thrives at 25 °C and responds with increasing CTmax to increasing acclimation temperatures, *D. subobscura* shows no clear positive acclimation response to increasing developmental temperatures and has reduced viability at 25 °C (Schou et al., 2017b). In accordance with our expectations we found the two species to differ substantially in the induction of the heat stress response, measured as inducible *hsp* expression, to both developmental acclimation and during thermal ramping. In *D. melanogaster* several genes showed a significant increase or a tendency for an increase in expression at higher developmental temperatures, which suggests that the heat stress response does play a role for developmental acclimation in this species. In *D. subobscura* the patterns were less clear, but for both *hsp23* and *hsp70* expression was reduced at 25 °C. We are not able to determine whether this reduction is the cause of the lack of beneficial acclimation in *D. subobscura* or whether it reflects that *D. subobscura* is severely stressed at 25 °C, but the low expression corresponds to the low acclimation capacity previously detected in this species (Schou et al., 2017b).

The two species also showed differences in their stress response during the ramping assays. First of all, the magnitude of the response was very modest in *D. subobscura* as compared to *D. melanogaster*. The maximal expression of *hsp23*, *hsc70-4,* and *hsp70* was approximately 2.5, 4, and 10-fold higher in *D. melanogaster* as compared to *D. subobscura*. The higher expression does not relate to a higher copy number (e.g. five copies of *hsp70* in *D. melanogaster* and two in *D. subobscura*), because both A and B clusters of *hsp70*, and in particular cluster A (which consists of two copies similarly arranged in the two species), shows the largest difference. A similar difference was detected for other *hsp* genes which do not show multiple copy numbers. Thus, upon heat stress *D. melanogaster* expresses higher amounts of *hsps* than *D. subobscura*.

Induction dynamics were also different between species. For *D. subobscura* we found a consistent increased expression with increasing temperature for two genes (*hsp23* and *hsp70*) and no effect of

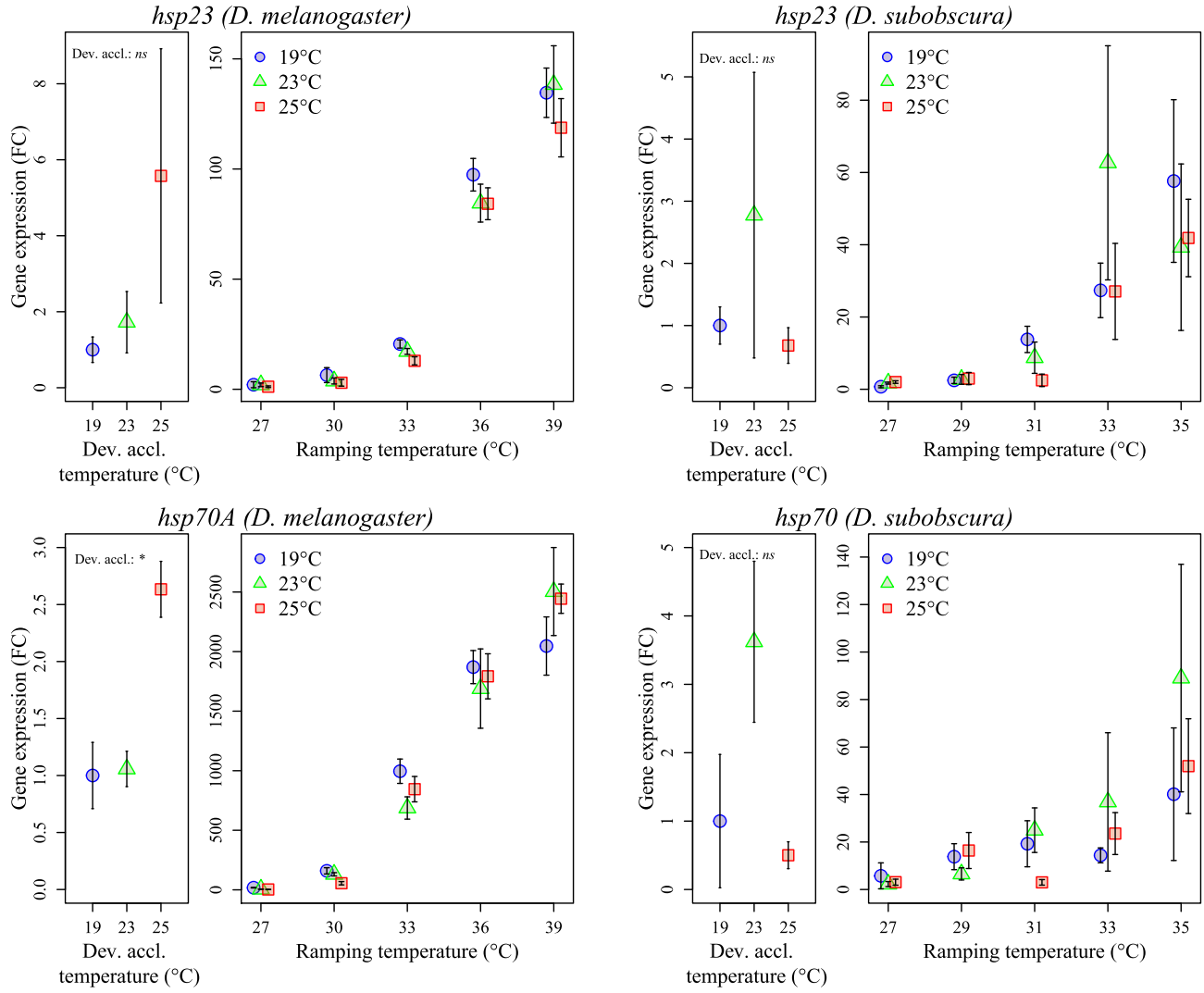

Fig. 2. Gene expression of *hsp23 and hsp70A/hsp70* in *D. melanogaster and D. subobscura, respectively.* For each gene the left panel shows expression level as a function of developmental acclimation temperature, while the right panel shows the expression level as a function of temperature (ramping at a rate of 0.1 °C/min from the developmental temperature). Expression levels are reported in fold change (FC) relative to the expression level of the 19 °C developmental acclimation (mean ± sem). Note that the Y-axes differ in scale between species. Statistical results for the linear effect of developmental acclimation for basal gene expression *: $P < 0.05$, ns: non-significant.

developmental temperature. For *D. melanogaster* the results were more complex. For two genes (*hsp70A* and *hsp83*) negative model estimates for developmental temperature were found (Table S3). Our interpretation is that the increase in expression with increasing ramping temperatures was stronger at lower developmental temperatures, i.e. a higher expression (and thus higher tolerance capacity) at higher developmental temperatures when exposed to mildly elevated temperatures. This in turn leads to a reduced expression (reduced need for repair) at a given stress temperature; and is in correspondence with the results of the basal expression at the developmental acclimation temperatures, where both genes also showed increased expression with higher acclimation temperature (although only for *hsp70A* significantly). Thus, an inverse relationship might exist between basal and severe stress induced levels of expression – a hypothesis that corroborates findings of high constitutive expression levels of *hsp70* in interspecific comparisons of non-*Drosophila* dipterans (Zatsepina et al., 2016). In support of this idea, the basal abundance of heat shock proteins can also show evolutionary change, after selection for heat and other types of environmental stress tolerance leads to changes in the basal abundance of heat shock proteins (Sørensen et al., 2017).

The interspecific differences in transcript accumulation upon thermal treatment observed in this study must reflect species-specific idiosyncrasies in the transcription regulation of the heat shock response. The molecular underpinnings of the heat shock response regulatory network are complex, typically integrating inputs from combinations of causal factors (reviewed in Garbuz, 2017; Sakurai and Enoki, 2010). Two key rate-limiting steps of heat-shock gene activation are i) establishment of a "potentiated" promoter through an orderly sequence of chromatin opening, transcription initiation and promoter-proximal pausing of RNA polymerase II; and ii) upon heat-shock, resumption of transcription elongation through release of the paused Pol II into the gene body (reviewed in Vihervaara et al., 2018). The two steps are respectively under coordinated control of two major transcription factors, specifically the GAGA factor (GAF), and the heat shock factor (HSF) (Duarte et al., 2016). In *Drosophila*, GAF and HSF are both encoded by single loci, (*Trl* and *Hsf*, respectively), which furthermore are also highly conserved, whereby variation in heat shock transcript accumulation should result primarily from variation in their respective transcription factors binding sites (TFBSs), namely GAGA sites (Bergman et al., 2005; Gilmour et al., 1989) and heat shock elements (HSEs; reviewed in Tian et al., 2010). GAGA-sites are GA/CT-rich sequences that can be located either interspersed or overlapping with

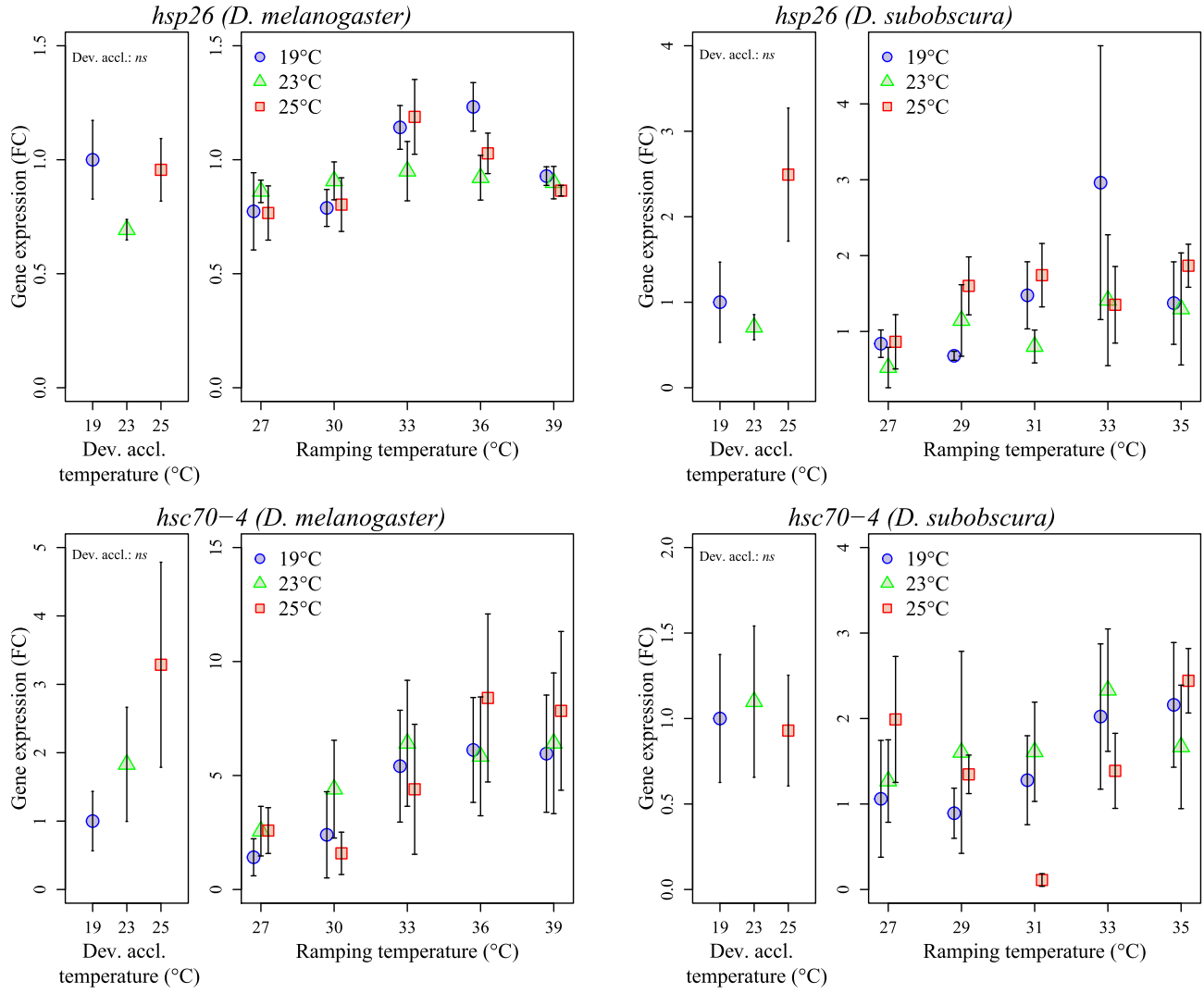

Fig. 3. Gene expression of *hsp26 and hsc70-4* in *D. melanogaster* and *D. subobscura*, respectively. For each gene the left panel shows expression level as a function of developmental acclimation temperature, while the right panel shows the expression level as a function of temperature (ramping at a rate of 0.1 °C/min from the developmental temperature). Expression levels are reported in fold change (FC) relative to the expression level of the 19 °C developmental acclimation (mean ± sem). Note that the Y-axes differ in scale between species.

Table 2
Results from the linear models for effect of developmental acclimation temperature and effects of ramping induced gene expression of four candidate genes in *D. subobscura.* The effect of ramping was evaluated by both a linear and a 2nd order polynomial component. See Tables S4 and S5 for the results of the full and final models after reduction. ***P < 0.001; ns not significant; - signifies that no test of significance was performed either because the full model was non-significant or because the interaction was significant.

	hsp23	*hsp70*	*hsp26*	*hsc70-4*
Ramping induced gene expression				
Ramping (linear/2nd order poly)	(***/ns)	(***/ns)	–	–
Developmental acclimation	ns	ns	–	–
Ramping x Dev. accl	ns	ns	–	–

HSEs. HSEs consist of at least three inverted repeats of the 5bp consensus motif nGAAn. The number of pentanucleotide motifs in individual HSEs (Xiao et al., 1991), the numbers and spacing between and within GAGA-sites and HSEs (Amin et al., 1987), and the ordering position and distances of the two types of TFBSs relative to the transcription start site (TSS) (Amin et al., 1988; Georgel, 2005) are all major determinants of the speed and magnitude of the heat-shock response. Either greater numbers of pentanucleotide motifs in individual HSEs, and/or greater numbers GAGA-sites/HSEs that are less spaced from each other and closer to the TSS (Topol et al., 1985) are expected to upregulate *hsp70* transcription (Amin et al., 1987, 1988; Georgel, 2005; Xiao et al., 1991). With this in mind, *D. melanogaster* could be anticipated to exhibit a stronger heat shock response than *D. subobscura* because i) its *hsp70* promoter is comparatively enriched in GAGA-sites (Fig. 4). Specifically, the canonical *hsp70* promoter of *D. melanogaster* contains five GAGA-sites (Bergman et al., 2005; Gilmour et al., 1989), whereas that of *D. subobscura* is predicted to contain only two [from a promoter scan with the FIMO tool (Grant et al., 2011) from the MEME suite (Bailey et al., 2015), using the *Drosophila melanogaster* Major Position Matrix Motif (DMMPMM) for *Trl* binding sites (Kulakovskiy et al., 2009) stored in the REDfly database (version 5.5.3; http://www.http://redfly.ccr.buffalo.edu/), and a p-value cutoff of 10^{-3}], which, in addition, are located far from the TSS; and ii) the two species have similar numbers of HSEs in their proximal promoters, but the former has one more pentanucleotide motif in HSE1 than the latter (Puig Giribets et al., 2019; Tian et al., 2010; see Fig. 4). Comparison with the outgroups *D. willistoni* and species of the subgenus *Drosophila* (Tian et al., 2010) indicates that this difference is ascribed to decay of the first pentanucleotide motif of HSE1 in *D. subobscura*. In addition to the two

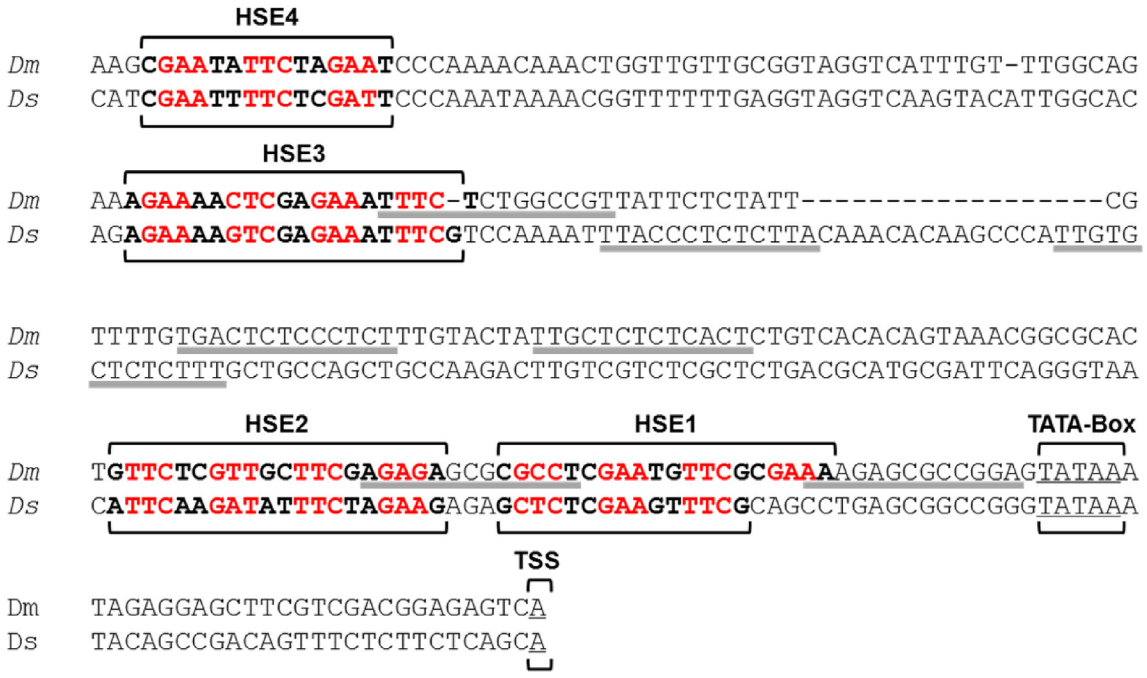

Fig. 4. Fig. 4. Schematic representation of a pairwise sequence alignment of the canonical *hsp70* proximal promoters of *D. melanogaster* (Gilmour et al., 1989; Tian et al., 2010) and *D. subobscura* (Puig Giribets et al., 2019), from the transcriptional start site (TSS) upstream to HSE4, showing the relevant TFBSs information, including GAGA-sites (underlined in grey) and HSEs 1–4 (bold faced and bracketed). For each HSE, core tri-nucleotides within pentanucleotides are colored in red. The alignment was conducted using the MAFFT's G–INS–i method (version 7; http://mafft.cbrc.jp/alignment/software/; Katoh and Standley, 2013) with default settings.

aforementioned differences, overall, *D. melanogaster* also exhibits a more compact proximal promoter, with TFBSs more shortly spaced, on average, than in *D. subobscura* (Fig. 4). Future studies could test the hypothesis that variation in the "strength" of promoters predict heat shock response intensity e.g. via *in vitro* assays. Such future studies could include intraspecific comparisons to investigate potential population-specific patterns. However, we did not include such intraspecific comparisons here; for genes with so high functional relevance we did not expect much variation to be present within species.

The comparison between the two species was challenged by the incompletely annotated transcriptome of *D. subobscura*, which meant we were not able to investigate as many genes in this species as in *D. melanogaster*. Still, our general prediction of a stronger heat shock response in *D. melanogaster* was confirmed. Further, no or negative regulation of the heat shock response at 25 °C acclimation in *D. subobscura* was in accordance with the lack of beneficial acclimation in this species, while in *D. melanogaster* we found indications of heat shock protein regulation to play a role for thermal adaptation at both benign (developmental acclimation) and during acute stress exposure (during ramping). These differences are likely attributable to documented differences in the genetic architecture of the promotor regions of these two species. In *D. melanogaster* we were able to differentiate among the A and B clusters of the *hsp70* genes. In accordance with a previous study we found different expression levels of the two clusters (Lakhotia and Prasanth, 2002). In this study only the A cluster showed a significant interaction between the effect of developmental acclimation and the effect of ramping, while the B cluster only showed a non-linear effect of ramping temperature. So far no clear understanding of a differentiated role for heat tolerance of these two clusters has been achieved, but the results of this study provide a first step and some useful results upon which new hypotheses can be tested.

Conflicts of interest

No competing interests declared.

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Data availability

The qPCR data presented in the current study are available at Mendeley Data: http://dx.doi.org/10.17632/f4h8tc5wz7.2 (Sørensen et al., 2019).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.07.005.

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