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Temporal specific coevolution of *Hsp70* and co-chaperone *stv* expression in *Drosophila melanogaster* under selection for heat tolerance

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ABSTRACT

Heat shock proteins (Hsps) have long been candidates for ecological adaptation given their unequivocal role in mitigating cell damage from heat stress, but linking Hsps to heat tolerance has proven difficult given the complexity of thermal adaptation. Experimental evolution has been utilized to examine direct and correlated responses to selection for increased heat tolerance in *Drosophila*, often focusing on the major Hsp family *Hsp70* and/or the master regulator HSF as a selection response, but rarely on other aspects of the heat shock complex. We examined *Hsp70* and co-chaperone *stv* isoform transcript expression in Australian *D. melanogaster* lines selected for static heat tolerance, and observed a temporal and *stv* isoform specific, coordinated transcriptional selection response with *Hsp70*, suggesting that increased chaperone output accompanied increased heat tolerance. We hypothesize that the coordinated evolutionary response of *Hsp70* and *stv* may have arisen as a correlated response resulting from a shared regulatory hierarchy. Our work highlights the complexity and specificity of the heat shock response in *D. melanogaster*. The selected lines examined also showed correlated responses for other measures of heat tolerance, and the coevolution of *Hsp70* and *stv* provide new avenues to examine the common mechanisms underpinning direct and correlated phenotypic responses to selection for heat tolerance.

1. Introduction

The ubiquitous expression of heat shock proteins (Hsps) during heat shock is so well characterized that this response serves as a general model of gene expression owed largely to the analyses of regulatory events at the heat-activated *D. melanogaster Hsp70* promoter (reviewed in Adelman and Lis, 2012; Chen et al., 2018). Hsps perform chaperone functions to stabilize proteins through conformational folding to protect cell homeostasis (Lindquist and Craig, 1988) and are rapidly activated by the binding of the master transcription factor heat shock factor (HSF) to heat shock sequence elements (HSEs) (discussed in Tian et al., 2010). Heat-activated Hsps are compelling candidates for ecological adaptation (Feder and Hofmann, 1999), and protein coding sequences are highly conserved among virtually all species examined reflecting an evolutionary history of strong purifying and ultimately stabilizing selection (reviewed in Sørensen, 2010; Sørensen et al., 2003). Hsp70 is the major inducible Hsp family in many organisms, and in *D. melanogaster* comprises the multi-copy inducible *Hsp70* gene organized in two clusters, A (two copies) and B (three copies) (Feder and Hofmann, 1999; Sørensen et al., 2003). The functional five-copy *D. melanogaster* Hsp70 family arose from amplification, with the fifth copy from tandem duplication and gene conversion, reflecting strong natural selection on duplicate genes to maintain enormous levels of induced Hsp70 expression over this species' expansive range (Bettencourt and Feder, 2001). Adaptive variation in Hsp70 and other Hsps however is less linked to copy number but rather to non-coding changes largely in *cis*-regulatory sequences (Sørensen et al., 2003), e.g. HSEs, which in *Drosophila* are seldom beyond 500 bp of the transcription start site (Tian et al., 2010).

The role of Hsp70 in improving survival during cellular heat shock is undisputed, but connecting this principal mechanism to thermal adaptation even for *Drosophila* remains elusive. Several reviews address the challenges of linking elevated Hsp levels to adaptive stress responses, from interpretation of Hsp expression and other trait measurements as markers of adaptation, the relevance of laboratory to field conditions, and the interaction of Hsps with other molecular and biological processes shaping life history events (Chen et al., 2018; Feder and Hofmann, 1999; Hoffmann et al., 2003; Sørensen, 2010; Sørensen et al., 2003). *Drosophila* exhibit contrasting life-cycle associations with Hsp70 and heat resistance e.g. increased Hsp70 appears important for increased larval heat resistance (Bettencourt et al., 2008; Krebs and Feder, 1998;

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Krebs et al., 1998) (but see Jensen et al. (2010)), but showed negligible association in adults (Dahlgaard et al., 1998; Jensen et al., 2010). Nonetheless, among diverse taxa, growing evidence suggests that global climate change interacts with Hsp expression in complex ways; although associations between Hsp expression and thermal adaptation are often indirect and subject to trade-offs in part due to high pleiotropy with 'cellular housekeeping' in the general handling proteins and in the degradation of misfolded or aggregated proteins (Chen et al., 2018; Sørensen, 2010). Costs incurred by stress activated Hsps are thought to occur through the toxic effects of high Hsp concentrations with high energy demands and interference with normal cell function (Sørensen, 2010).

Experimental evolution provides opportunity to study direct and correlated responses to selection and has been widely used in *Drosophila* for understanding the evolution of adaptive heat tolerance (discussed in Hangartner and Hoffmann, 2016). Debate surrounds the methodologies used to study adaptation to hyperthermia given the complexity of heat tolerance and whether different measures of heat tolerance share a genetic basis or are separate traits (Hangartner and Hoffmann, 2016; Sørensen, 2010). Hangartner and Hoffmann (2016) addressed this issue by testing for direct and correlated responses in different heat tolerance assays following selection for exposure to static high temperature in Australian *D. melanogaster*. Heat tolerance is often measured by scoring knockdown time using a static stressful temperature (Hoffmann et al., 2002) or by gradually increasing temperature (ramping method), which is thought to be more ecologically appropriate (Terblanche et al., 2011). More recently Jørgensen et al. (2019) demonstrated the ecological relevance of both static and dynamic assays which correlate with low precipitation and maximum temperature in *Drosophila* species adapted to different climates. Hangartner and Hoffmann (2016) found direct responses to selection for increased static knockdown, and correlated responses in ramping and other measures of heat tolerance suggesting a shared genetic basis and a common mechanism affecting heat tolerance, regardless of whether static or ramping measures of tolerance are considered.

Hsp70 expression in *Drosophila* lines selected for heat tolerance has also been examined as a measure of adaptation to elevated temperature, although selection led to decreased inducible Hsp70 expression among different species, interpreted as the costs of high Hsp70 expression outweighing the benefits under recurrent stress (discussed in Sørensen et al., 2003). Selected lines founded from Australia increased Hsp70 levels consistently throughout selection, although on average only 15% higher than controls, again suggesting the constraints of pleiotropy (Feder et al., 2002). Genome-wide studies have demonstrated the breadth of genes transcriptionally reprogrammed by heat shock, surpassing Hsps in terms of number, function and regulatory variation (Vihervaara et al., 2018), including non-Hsp genes with HSF binding sites (Gonsalves et al., 2011). This highlights the complex path from the cellular heat shock response to thermal tolerance, but also expands greatly on evolutionary possibilities contributing to thermal adaptation. The sole *Drosophila* orthologue of the human Hsp70 co-chaperone BAG proteins, *starvin* (*stv*) (Coulson et al., 2005) gene is regulated by HSF (Gonsalves et al., 2011; Guertin and Lis, 2010; Jensen et al., 2008) and up-regulated during heat shock and recovery and other stresses with life-cycle, genotype and geographic specificity (discussed in Telonis-Scott et al., 2014). We have shown that different isoforms of this complex locus are expressed as three discrete thermal and temporal RNA phenotypes, whereby alternative promotor usage of the shorter isoforms are favored under heat shock and the shortest isoform is exclusively heat inducible in adult *D. melanogaster* (Telonis-Scott et al., 2013, 2014).

Altogether, these observations suggest a basis for regulatory genetic variation at the *stv* locus, which encodes products that are Hsp binding (Carra et al., 2010). BAG co-chaperone proteins bind to heat induced Hsp70 and the constitutively expressed cognate Hsc70 proteins to modulate chaperone activity (reviewed in Arndt et al., 2007). Further, we demonstrated that the long *stv* transcript isoforms differ in expression to different thermal treatments between temperate and tropical east Australian populations with different heat tolerances under common garden conditions (Telonis-Scott et al., 2014). The combination of regulatory variation and the transcriptional flexibility of *stv* expression during heat shock make this gene an appealing candidate contributing to the complex trait of heat tolerance in *D. melanogaster*. To date, natural and experimental evolution studies have focused on either Hsp70 and/or HSF, but not *Hsp70* and co-chaperone evolution. To address this, we utilized the Australian experimental evolution lines of Hangartner and Hoffmann (2016) to test if expression of the inducible *Hsp70A* gene cluster and co-chaperone *stv* transcript isoform expression change in response to selection for static heat knockdown. We examined *stv* isoform transcript levels as a selection response during heat shock and recovery given the locus encodes seven transcripts and five unique proteins, all of which contain the BAG domain, with no other known domains in the variable regions suggesting common protein isoform function (Telonis-Scott et al., 2014). Moreover, *Hsp70* transcript levels track with Hsp70 protein expression (Bettencourt et al., 1999), providing a useful marker of gene regulation during heat shock. We hypothesize that coevolution of *Hsp70* and *stv* expression under selection for heat resistance (either higher or lower expression in the selected lines) would provide evidence for a shared genetic basis at the molecular level leading to new lines of enquiry for understanding the common mechanisms of direct and correlated selection measures of heat tolerance.

2. Materials and methods

2.1. *D. melanogaster* selection lines and heat knockdown assays

The experimental evolution lines from Hangartner and Hoffmann (2016) were founded from a *D. melanogaster* population established from 60 isofemales collected near Melbourne, Australia in May 2012. Briefly, flies were mass-bred for two generations in the laboratory prior to the first round of selection at generation F_3 (19 °C, under a 12:12 h light: dark cycle). For the first selection experiment, 5000 virgin males and females were separated into groups of 25 and allowed to mature until 4–8 days old. A static heat knockdown assay was used for selection by immersing 100 ml glass bottles containing 100 flies in a circulating water bath set at 39 °C. The bottles were removed from the water bath when approximately 90% of flies were knocked down. The non-comatose flies (upright and moving) were selected and randomly allocated into five replicate lines (S1–S5). The selection assay was performed separately on the sexes, and each replicate line was founded from 90 to 110 flies of each sex. The lines were selected in this manner for eight consecutive generations, (selection intensity of 10% on late knockdown, the non-comatose 100 selected out of 1000 flies per sex) then relaxed to every second generation until generation 21 for a total of 14 selection generations. The control lines were established by randomly allocating an equivalent number of generation F_3 males and females as the selected flies into five replicate lines (C1–C5) (Hangartner and Hoffmann, 2016). The random establishment of the control lines does not permit direct comparisons between individual control and selected lines, as the numbering of the lines does not indicate pairs but rather independent replicates of either the selected or control treatment.

We assessed four of the five replicate lines (S1–S4 and C1–C4) for heat knockdown time and *Hsp70A* and *stv* isoform transcript expression. The lines were cultured at 20 °C on potato-dextrose -agar media, under a 12:12 h light: dark cycle. Experimental flies were collected within 24 h eclosion, matured for 4–5 days then separated by sex using by manual aspiration into groups of 20, and allowed 24 h for handling recovery. Thermotolerance was assessed using a static heat knockdown assay at generation F_{24}. For the assays, individual six-day-old male and female flies were placed into 5 ml waterproof vials and immersed into a circulating water bath heated to 39 °C. Heat knockdown time was scored as the time taken (to the nearest second) for individual flies to be

immobilized. Flies were assessed over four replicate blocks performed on the same day containing approximately 8 flies from each sex/treatment/replicate line for a total of approximately 32 flies per sex/treatment/replicate line combination (512 flies in total).

2.2. Time-course sampling during heat stress and recovery for transcript measurements

Preliminary analysis of the phenotypic data showed no differences between the sexes, thus females were assessed for gene expression to maximize replicates and time-points. To profile transcript expression during stress and in recovery, a time-course was determined (Telonis-Scott et al., 2013). Briefly, flies were placed in a circulating water bath heated to 39 °C and exposed to increasing increments of heat stress and mortality was monitored in the first 48 h following exposure. Both the control and selected lines withstood 22 min of heat shock without mortality and this was chosen as the maximum heat exposure prior to recovery in the time-course. Flies were reared and collected as for the heat knockdown assays. Groups of 20 six-day old F_{25} females were placed into 15 mL Bunzel cryotubes and snap frozen in liquid nitrogen after the following treatments: immediately pre-stress (20 °C, on potato-dextrose -agar media); during water bath heat exposure (39 °C, no media) at 10 min and 22 min (referred to as 0.17 h and 0.37 h respectively for visual purposes); during the recovery period (20 °C, on media) following the maximum heat stress (39 °C for 22 min) at 1, 4, and 8-h post-exposure. Three independent biological replicates of 20 flies were sampled at each time-point per replicate line (144 samples in total).

2.3. RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from 20 flies per sample using a modified Trizol Reagent (Ambion) protocol. Following collection of the aqueous phase, 0.5 volume of 100% ethanol was added and the isolation was completed using a Roche High Pure RNA Tissue Kit, following the manufacturer's instructions. RNA yield and purity was determined by spectrophotometry (Nanodrop Technologies). RNA integrity was assessed with 1% agarose gel electrophoresis. cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction volume to using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions using the anchored-oligo $(dT)_{18}$ primer. The cDNA was diluted 1:10 in water. Real time PCR was performed in a 10 μl volume using a *Roche* Light Cycler® 480 and SYBR® Green chemistry. Reactions were performed in duplicate for each cDNA sample, with three biological replicates for each treatment/replicate line combination. *stv-RF:RA:RE ştv-RG:RB:RC, stv-RD* and *RPL11* transcripts were amplified using previously designed primers (Telonis-Scott et al., 2014). Primers were designed to amplify transcript subsets due to low sequence complexity at the exon junctions of the long and mid transcripts *stv-RF:RA:RE* and *stv-RG:RB:RC* (Telonis-Scott et al., 2014). *Hsp70A* transcripts were amplified with the following primers, forward: TCGATGGTACTGACCAAGATGAAG, reverse: GAGTCGTTGAAGTAGGCTGAA. Transcript abundance was normalized to the thermally and temporally stable gene *RPL11* (Telonis-Scott et al., 2013, 2014) where normalized expression of the transcript of interest $\mathbf{(TOI) = 2^{(RPL11\text{-}TOI)}}$.

2.4. Statistical analysis

2.4.1. Heat tolerance phenotypic data

Differences in heat resistance, measured as mean knockdown time between selected and control lines were assessed with a 3-factor mixed model analysis of variance (ANOVA) with the fixed effects of selection (selected or control lines), sex and block. Variation within replicate lines was accounted for in the model by nesting replicate lines in selection as a random effect. The interaction between selection and sex was examined to determine if the selection response was sex-specific.

2.5. Transcript expression data

The transcript expression data were log2 transformed for normality. The fixed effects of selection and time and the random nested term of replicated lines within selection were assessed using a 3-factor mixed model ANOVA for each transcript/s. The interaction between selection and time was used to examine whether the transcript-level selection response was temporally dependent. Planned contrasts for each transcript/s were employed to test if mean transcript levels differed between the control and selected lines. Tukey-Kramer *post-hoc* tests for unequal sample sizes were employed where a significant selection-by-time-point interaction was found. For visual interpretation, fold changes relative to time-zero (pre-stress) were calculated for each line using the median of the three time zero replicates for each replicate line/treatment/transcript/s, as groups of flies from each line were randomly allocated for the assays and were not matched. All analyses were performed using SAS software version 9.4 (SAS Institute, Cary).

3. Results

3.1. Independent verification of the heat knockdown selection response

Three-way mixed model nested ANOVA showed a significant effect of selection for heat knockdown resistance (Table 1, Fig. 1), however this did not differ between the sexes either in the fixed effects of sex, or sex-by-selection interaction term (Table 1). The block effect was significant, but this also did not differ between the sexes therefore the model was reduced to remove the block-by-sex interaction term. Variation between replicate control and selected lines was significant and accounted for in the model with the random term of replicate line nested within selection (Table 1.).

3.2. ANOVA results for Hsp-70A, and stv isoforms

Three-way mixed model nested ANOVA were fit for each transcript/subset separately. The main effect of time-point was significant for the expression of all transcripts (*Hsp-70A*, *stv-RG:RB:RC*, *stv-RF:RA:RE ştv-RD*, Table 2) and the selection-by-time interaction was significant for *Hsp-70A* and the middle isoform subset *stv-RG:RB:RC* expression (Table 2). Both *Hsp-70A* and the shortest transcript *stv-RD* showed significant line variation expression, and there was no significant effect of the selection term for any transcript/subset (Table 2).

3.3. Coevolution of Hsp70, stv-RG:RB:RC and stv-RF:RA:RE expression in response to selection for heat tolerance

Planned contrasts of the selected and control lines for each transcript/s and time-point (pre-stress, 0.17- and 0.37-h heat stress at 39 °C, and 1, 4 and 8-h recovery at 20 °C) were significant for *Hsp-70A* at pre-stress and 0.17-h stress, where basal least squares mean expression was lower in the selected lines but higher than the controls in early heat stress (selected *vs* control 0-h, 0.17-h, $P < 0.05$, Fig. 2A). Expression was significantly higher in the selected lines at 4-h recovery for both *Hsp70A*,

Table 1
Three-way mixed model nested ANOVA results for the fixed effects of selection (control or selected lines), sex, and block and random term of replicate line nested within selection for average heat knockdown time (in minutes). Terms significant at $\alpha = 0.05$ are shown in bold.

Main effects	*d.f.*	Type 111 SS	F Value	*P* Value
Selection	1	42,772	35.5	**0.001**
Sex	1	264.0	1.84	0.17
Block	3	2353.7	5.46	**0.001**
Selection*sex	1	253.80	1.77	0.18
Line(Selection)	6	7218.4	8.37	**<0.0001**
Error	488	70,138		

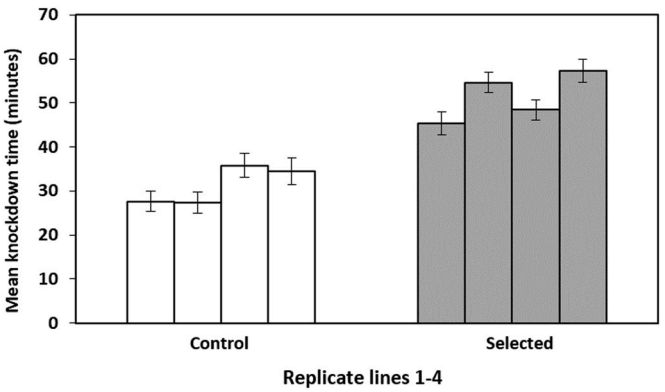

Fig. 1. Average heat knockdown time of individual *D. melanogaster* females from the control treatment (white bars) and selected treatment (grey bars), replicate lines 1–4. The treatments are shown separately as the lines are unpaired independent replicates from the founding population. The selection regime significantly increased knockdown resistance compared to the controls (3-way mixed model ANOVA, $P < 0.001$), and there was significant line variation (nested in treatment, $P > 0.001$). There was no effect of sex, and males are not shown. Error bars represent ± SE of the mean.

Table 2
Three-way mixed model nested ANOVA results for the fixed effects of selection (control or selected lines) and time-point, and random term of replicate line nested within selection for *Hsp70A* and *stv* isoform transcript expression. Terms significant at $\alpha = 0.05$ are shown in bold.

Main effects	*d.f.*	Type 111 SS	F Value	*P* Value
Hsp70A				
Selection	1	0.72	1.33	0.29
Time	5	1660.8	2415.8	**<0.0001**
Selection*Time	5	2.83	4.13	**0.002**
Line(Selection)	6	3.28	3.98	**0.001**
Error	123	16.9		
stv-RG:RB:RC				
Selection	1	1.89	3.92	0.09
Time	5	844.2	404.5	**<0.0001**
Selection*Time	5	5.19	2.49	**0.03**
Line(Selection)	6	1.82	0.73	0.63
Error	123	51.3		
stv-RF:RA:RE				
Selection	1	0.791	1.20	0.31
Time	5	44.60	21.65	**<0.0001**
Selection*Time	5	3.55	1.71	0.14
Line(Selection)	6	3.94	1.58	0.15
Error	123	51.07		
stv-RD				
Selection	1	0.327	0.20	0.67
Time	2	190.2	161.6	**<0.0001**
Selection*Time	2	0.40	0.34	0.71
Line(Selection)	6	9.75	2.76	**0.02**
Error	58	34.14		

the middle isoform subset *stv-RG:RB:RC*, and the long isoform subset *stv-RF:RA:RE* (*Hsp7A*selected *vs* control 4-h, $P < 0.05$, *stv-RG:RB:RC* selected *vs* control 4-h, $P < 0.001$, *stv-RF:RA:RE* selected *vs* control $P < 0.05$, (Fig. 2A,B,C). In agreement with previous findings, *stv-RD* expression was exclusively heat inducible (Telonis-Scott et al., 2013, 2014), and not reliably detected using qPCR until 1-h recovery. There were no differences in *stv-RD* expression between the selected and control lines at 1-, 4- and 8-h recovery (Fig. 2D).

3.4. Temporal-dependent coevolution of transcript expression

Hsp70A and *stv-RG:RB:RC* were further examined using Tukey-Kramer *post-hoc* pair-wise comparisons. To make meaningful inferences of temporal expression patterns from the large number of tests for each line/transcript/subset we focused on the *within* line comparisons. For *Hsp70A* the temporal profiles of both selected and control lines were similar; rapidly inducible during heat stress with peak expression at 4-h recovery from stress (selected pre-stress *vs* selected 0.17- and 0.37-h heat stress, and 1, 4 and 8-h recovery $P < 0.0001$ for all comparisons; control pre-stress *vs* control 0.17- and 0.37-h heat stress, and 1, 4 and 8-h recovery $P < 0.0001$ for all comparisons, Fig. 3A). Peak expression at 4-h recovery was significantly higher than all time-points during stress and recovery (selected 4-h recovery *vs* selected 0.17- and 0.37-h heat stress, and 1- and 8-h recovery $P < 0.0001$ for all comparisons; control pre-stress *vs* control 0.17- and 0.37-h heat stress, and 1, 4 and 8-h recovery $P < 0.0001$ for all comparisons, Fig. 3A). By 8-h recovery mean *Hsp70A* expression in both selected and control lines decreased and were not different from 1-hr recovery expression levels.

The temporal profiles of *stv-RG:RB:RC* expression were also alike in the selected and control lines; increasing in late heat stress with peak expression at 4-h and 8-h recovery from stress (selected pre-stress *vs* selected 0.37-h heat stress $P < 0.01$, and 1, 4 and 8-h recovery $P < 0.0001$; control pre-stress *vs* control 0.37-h heat stress $P < 0.001$, and 1-, 4- and 8-h recovery $P < 0.0001$, Fig. 3B). Expression at 4- and 8-h recovery was higher than late heat stress and 1-h recovery (selected 4-h recovery *vs* selected 0.37-h heat stress, and 1-h recovery $P < 0.0001$ for all comparisons; control 4-h recovery *vs* control 0.37-h heat stress, and 1-h recovery $P < 0.0001$ for all comparisons; selected 8-h recovery *vs* selected 0.37-h heat stress, and 1-h recovery $P < 0.0001$ for all comparisons; control 8-h recovery *vs* control 0.37-h heat stress, and 1-h recovery $P < 0.0001$ for all comparisons Fig. 3B). In contrast to *Hsp70A* there were no expression differences between 4- and 8-h recovery in either selected or control lines. Expression relative to pre-stress is shown for *stv-RF:RA:RE* and relative to 1-h recovery for *stv-D* (Fig. 3C and D).

Based on these temporal profiles the terms largest shifts in the selected lines were in mean increases in transcript abundance for *Hsp70A* and *stv-RG:RB:RC* at 4-h post recovery. Average fold-changes relative to pre-stress were 740- and 48-fold higher in the selected lines compared to controls at 4-h recovery for *Hsp-70A* and *stv-RG:RB:RC* respectively (Fig. 2A and B.).

4. Discussion

Hangartner and Hoffmann (2016) found correlated responses for multiple measures of heat tolerance in Australian *D. melanogaster* selected for increased static heat knockdown, reflecting some common genetic architecture for varied traits. In the same lines, we have shown that two key heat shock response genes also evolved higher transcript abundance in a temporal-dependent manner during heat stress and recovery. *Hsp70* gene products in *Drosophila* lines selected for heat tolerance have been studied as heat adaptive traits, often with mixed outcomes (Sørensen et al., 2003). We examined the combination of *Hsp70A* and co-chaperone *stv* isoforms and found transcriptional coordination of *Hsp70A* and *stv* middle *-RG:RB:*RC isoforms at 4-h recovery from heat stress. Both *Hsp70A* and *stv* middle and short isoforms are heat inducible, and reach peak expression during the recovery repair phase (Colinet and Hoffmann, 2010; Telonis-Scott et al., 2013, 2014). Here, selection resulted in temporal specificity with the largest expression increases occurring at 4-h recovery compared to the control lines, although *Hsp70A* transcripts also increased at the critical point of early heat onset. The long *stv-RF:RA:RE* isoforms were weakly inducible only in recovery and also showed a modest coordinated response at 4-h recovery, however we demonstrate that *stv-RG:RB:*RC transcripts are preferentially transcribed during heat shock and respond with greater transcriptional capacity to track recovery levels of *Hsp70* abundance under selection for heat tolerance.

We examined heat selected lines that harbor genetic variation to increase heat tolerance, but like other directional selection studies these lines reached a phenotypic plateau (Berrigan et al., 1997; Gilchrist and Huey, 1999) after 8–10 generations of selection, and thus limiting the

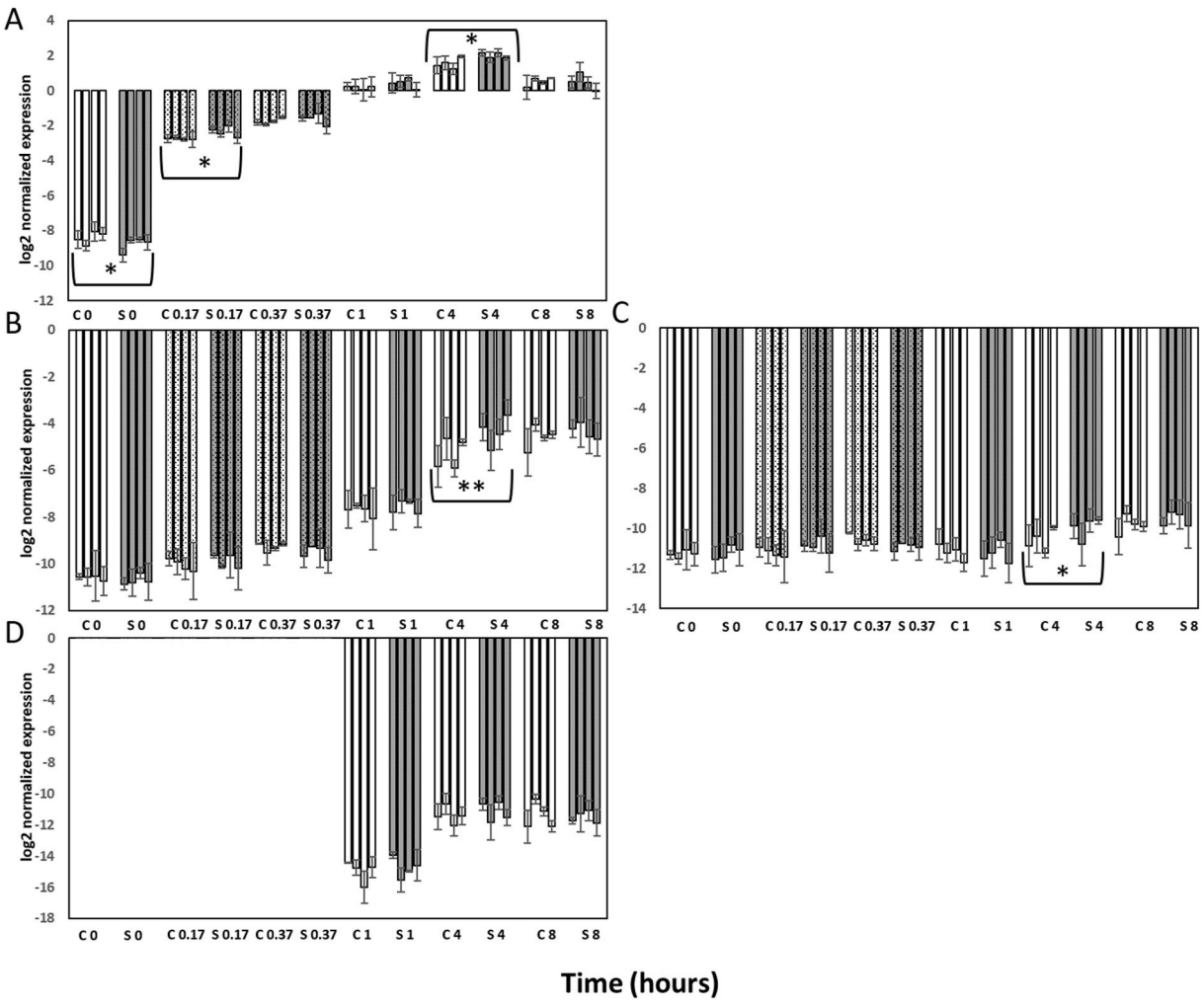

Fig. 2. *Hsp70A* and *stv* isoform log2 normalized expression pre-stress, during heat stress and recovery. White bars = control lines, grey bars = selected lines. Dotted bars = heat stress (39 °C), open bars = recovery (20 °C) following heat exposure for 22 min. Asterisks indicate significant time-point differences between the selected and control lines (log2 normalized expression least squares means, planned contrasts). The treatments are shown separately as the lines are unpaired independent replicates from the founding population (C = control, S = selected). **A.** *Hsp70A* expression. Basal (pre-stress) levels of *Hsp70* were 1.3-fold lower in the selected lines compared to the controls, while expression at early stress 10 min and 4-h recovery was 1.3- and 1.4 higher in the selected lines respectively. **B.** *stv-RG:RB:RC* middle isoform subset. Expression of *stv-RG:RB:RC* isoforms at 4-h recovery were on average 1.9-fold higher in the selected lines compared to the controls. **C.** *stv-RF:RA:RE* long isoform subset. Expression of *stv- RF:RA:RE* isoforms at 4-h recovery were on average 1.5-fold higher in the selected lines compared to the controls. **D.** *Stv-D* short isoform expression. *Stv-D* transcripts did not accumulate in all lines until 1-h recovery and did not differ between the control and selected lines.

increase in resistance to around 0.5 °C (Hangartner and Hoffmann, 2016). Drosophilid species are limited in their capacity to evolve higher heat tolerance, perhaps in part to evolutionary constraints on the inducible heat shock machinery (Kellermann et al., 2012), but less is known about how these mechanisms are subject to natural selection. *Drosophila* are characterized by inducible *Hsp70* expression upon heat shock and degradation of *Hsp70* gene products during later recovery, but emerging data from non-model dipterans and other taxa from contrasting habitats show that variation and stability in constitutive *Hsp70* expression is a common adaptive response (Zatsepina et al., 2016). We consistently detected very low level basal (pre-stress) *Hsp70* transcripts in both control and selected lines, and found slightly lower basal expression of *Hsp70* following selection, but observed the largest changes in inducible expression during early stress and at 4 h recovery. Decreasing basal *Hsp70* expression may be a trade-off for evolving higher and costly inducible expression (Sørensen et al., 2003) in the selected lines.

Nonetheless, *D. melanogaster* exhibit historical and short-term capacity to evolve various regulatory targets on which selection could act to alter inducible *Hsp70* expression. Recently, *Hsp70* promoter architecture was proposed to play a role in interspecific variation for increased heat tolerance following acclimation, where higher thermotolerance and stronger Hsp response was observed in tropical derived *D. melanogaster* compared to temperate *D. subobscura*, as predicted by sequence feature comparison of the more rapidly induced and prolific *D. melanogaster* promoter (Sørensen et al., 2019). Long term laboratory thermal acclimation of *D. melanogaster* revealed evolutionary changes in Hsp70 expression due to regulatory variation rather than gene copy number (Bettencourt et al., 1999), including shifts in HSF protein activation (Lerman and Feder, 2001). Despite the trade-offs reported for increasing *Hsp70* under selection, we and Feder et al. (2002) found increased transcripts and protein respectively in Australian founded selected lines. This, together with the coevolution of the most heat responsive *stv* transcripts suggests that at least some aspects of the heat shock machinery also show evolutionary capacity to increase chaperone output concomitant with increased heat tolerance in *D. melanogaster*. The complexity and pleiotropy of genes involved in the heat shock response, and the organismal phenotypic limits however indicate evolutionary constraints at least in *Drosophila*.

We propose that rather than selection acting separately on *Hsp70* and

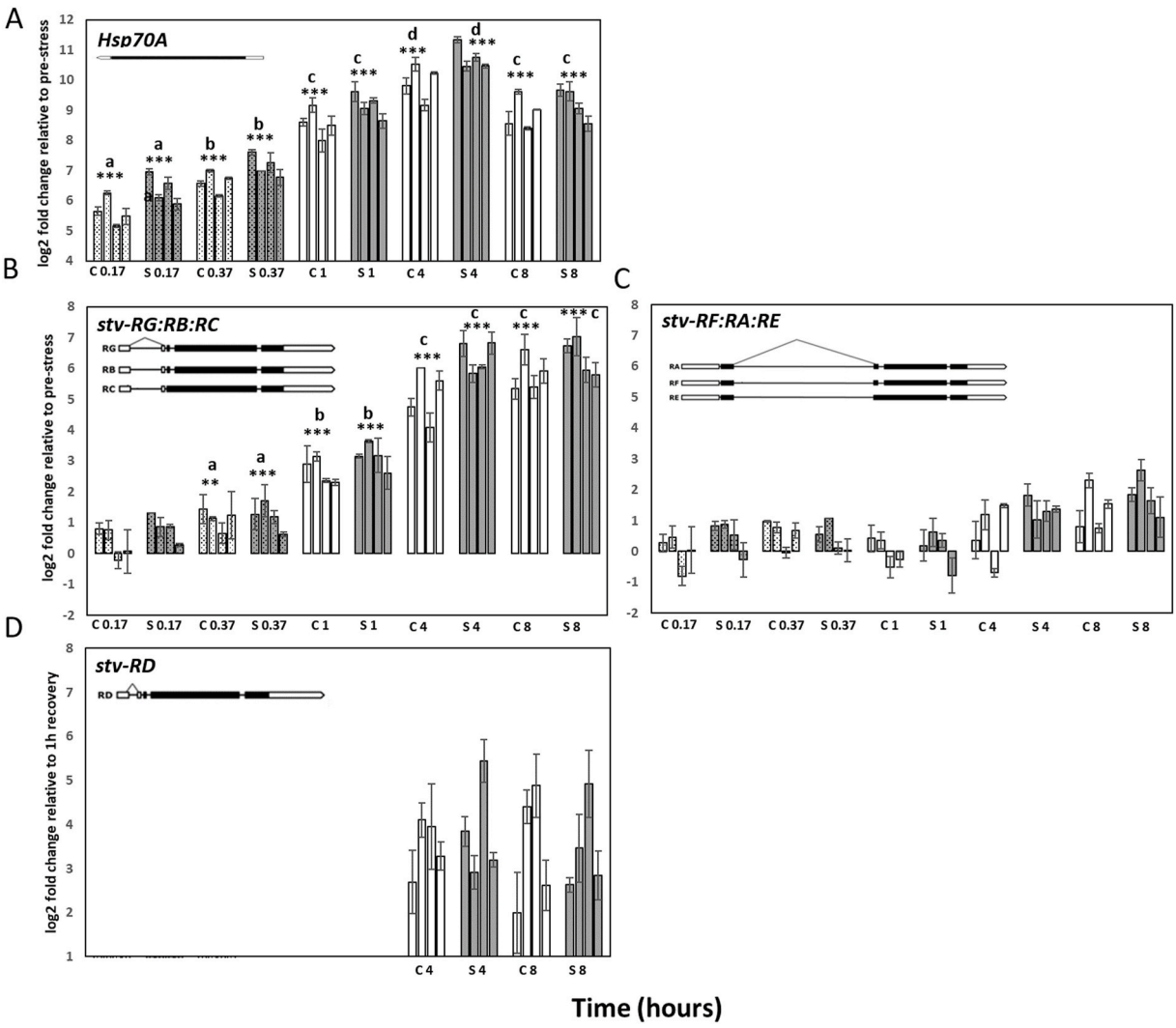

Fig. 3. *Hsp70A* and *stv* isoform log2 fold-change relative to pre-stress during heat stress and recovery. White bars = control lines, grey bars = selected lines. Dotted bars = heat stress (39 °C), open bars = recovery (20 °C) following heat exposure for 22 min. The transcript/transcript subset structure are shown in the top left hand of each panel (not to scale, adapted from Flybase *v* 2020_06). Asterisks above each set of lines indicate significant within treatment expression differences relative to pre-stress, and letters indicate significant differences between time-points (Tukey-Kramer *post-hoc* comparisons). The treatments are shown separately as the lines are unpaired independent replicates from the founding population (C = control, S = selected). **A.** *Hsp70A* expression relative to pre-stress. *Hsp70A* was rapidly heat inducible in both selected and control lines with peak expression at 4-h recovery. Average *Hsp70A* expression relative to pre-stress was 33- and 740-fold higher in the selected lines compared to the control lines at 0.17-h stress and 4-h recovery respectively. **B.** *stv-RG:RB:RC* middle isoform subset. The middle *stv* isoforms were also heat inducible from late stress in both selected and control lines peaking at 4- and 8- recovery. The middle subset was expressed on average 2-fold higher at late heat stress relative to pre-stress. Average selected treatment *stv-RG:RB:RC* isoform expression was 48-fold higher than the control lines at 4-h recovery. **C.** *stv-RF:RA:RE* long isoform subset. The *stv* long isoforms did not accumulate relative to pre-stress until 4- and 8-h recovery with fold-changes on average 2-fold higher. The selected lines upregulated *stv-RF:RA:RE* on average 1.1-fold higher than the control lines at 4-h recovery. D. *stv-RD* expression is shown relative to 1-h recovery when transcripts were reliably detected. There were no differences between the selected and control lines. Error bars represent ± SE. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

stv, these coordinated shifts result from a shared regulatory hierarchy. The rapid turnover of heat-induced genes requires accessible chromatin, and in *D. melanogaster,* GAGA-associated factor (GAF) which recognizes GAGA-rich sequences is vital in maintaining the nucleosome-free chromatin resulting in an open promotor state prior to stress (Duarte et al., 2016). GAF also maintains the pausing of RNA Polymerase II (Pol II) at heat-activated promoters, the major transcriptional regulatory step via Pol II promoter-proximal pause and release into elongation (reviwed in Vihervaara et al., 2018). The pause release is modulated by the trimerisation of inactive monomers through interactions with chaperones of the master transcription factor Heat shock factor (HSF), which binds DNA and recruits chromatin remodelers and positive transcription factors (reviwed in Vihervaara et al., 2018). HSF regulates the rapid transcription of Hsps, but heat-activated-genes can be independent of HSF binding (Duarte et al., 2016), while many heat-activated genes are bound by HSF but are not Hsps (Gonsalves et al., 2011). The Hsp70 model of Hsp regulation however is generalizable to most heat-responsive genes (Vihervaara et al., 2018).

Evidence from genome-wide studies supports a Hsp mode of transcriptional regulatory control of *stv*, providing a plausible co-evolutionary pathway with *Hsp70*. Lee et al. (2008) identified a peak of GAF within 1 kb of the *stv* transcription start site (TSS) using Chromatin immunoprecipitation (ChIP) microarray assays to identify protein-DNA interactions. Fuda et al. (2015) utilized Global Run-on sequencing (GRO-seq) to map nascent transcripts from engaged Pol II, and identified *stv* promotor bound GAF. GAF is enriched on promoters with paused Pol II, and Duarte et al. (2016) demonstrated via Precision Run-On Sequencing that *stv* is a heat activated gene and maintains

promoter-proximal stalled Pol II across the gene body prior to heat shock. Taken together, this suggests that for heat shock induced genes including *stv*, GAF binding close to the TSS prior to heat exposure is important for rapid gene accessibility and heat activation (Duarte et al., 2016). We observed a transcriptional lag and lower induced expression compared to *Hsp70* despite the expression of the middle *stv* isoforms during late heat stress and rapid accumulation during recovery. The *Hsp70* promotor is more rapidly heat induced than *stv* likely due to the more prolific HSEs, and *stv* interacts with Hsc70 proteins to modulate chaperone activity. *Stv* transcripts do not track *Hsp70* during heat stress but play a larger role at 4-h recovery, perhaps in modulating *Hsp70* levels after 4-h recovery as *stv-RG:RB:RC* transcripts remained elevated at 8-h recovery.

Still equivocal is how the middle *stv* transcript isoforms are preferentially activated during heat stress, and we suggest this may be associated with increased recruitment of HSF proximal to the *stv-RG:RB:R*C TSS as opposed to the longer isoforms, based on HSE sequence analysis (Telonis-Scott et al., 2014) and experimental HSF binding evidence during heat shock (Gonsalves et al., 2011; Guertin and Lis, 2010). We consistently detect mature *stv-RG:RB:R*C transcripts during late heat shock, leading to peak expression at 4-h recovery corresponding with *Hsp70A*. Recent discoveries show that transcription elongation and nascent RNA processing events including intron splicing occur concurrently and involve cross-talk between chromatin, transcription and splicing (reviwed in Herzel et al., 2017). Heat shock of *Mus musculus* fibroblast cells caused intron retention in 27% of transcripts with at least two introns, while heat-induced genes continued to be spliced due to co-transcriptional splicing, including BAG1 (Shalgi et al., 2014), a mouse co-chaperone for Hsp70/Hsc70. This supports the importance of BAG genes during heat shock and recovery and provides a probable mechanism for *stv* transcript processing during heat shock.

In conclusion, both *Hsp70* and co-chaperone *stv* expression show evolutionary capacity to increase expression in response to selection for increased static heat knockdown in Australian *D. melanogaster*. The temporally coordinated recovery response was *stv* isoform specific, supporting the transcriptional preference of heat responsive 'middle' *stv* isoforms *stv-RG:RB:R*C during heat stress and recovery. The tracking of a co-chaperone with *Hsp70* activity over time suggests that in these lines, the heat shock machinery likely increased chaperone activity with increasing heat tolerance. Although this requires examination at the protein level, such a mechanism is indicated by the congruence between *Hsp70* expression and protein levels. Apart from another set of Australian selected lines (Feder et al., 2002), this outcome conflicts with the trade-offs associated with elevated *Hsp70* expression commonly observed (Sørensen et al., 2003). Whether this is particular to Australian *D. melanogaster* is unknown, but fecundity and survival in the selected lines was not different to the controls (Hangartner and Hoffmann, 2016). We cannot preclude constraints on increasing *Hsp70* and *stv* expression as we measured expression at the end of selection rather than progressively during selection, but the observed phenotypic plateau in heat tolerance (Hangartner and Hoffmann, 2016) suggests limits at the molecular level, consistent with the physiological limits in heat tolerance. By examining a Hsp and co-chaperone, we hypothesize that the coordinated evolutionary response may have arisen as a correlated response resulting from a shared regulatory hierarchy, providing new avenues to examine the common mechanisms underpinning direct and correlated phenotypic responses to selection for heat tolerance.

Author statement

The author's declare no conflict of interest.

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