Spatial analysis of gene regulation reveals new insights into the molecular basis of upper thermal limits

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Abstract

The cellular stress response has long been the primary model for studying the molecular basis of thermal adaptation, yet the link between gene expression, RNA metabolism and physiological responses to thermal stress remains largely unexplored. We address this by comparing the transcriptional and physiological responses of three geographically distinct populations of Drosophila melanogaster from eastern Australia in response to, and recovery from, a severe heat stress with and without a prestress hardening treatment. We focus on starvin (stv), recently identified as an important thermally responsive gene. Intriguingly, stv encodes seven transcripts from alternative transcription sites and alternative splicing, yet appears to be rapidly heat inducible. First, we show genetic differences in upper thermal limits of the populations tested. We then demonstrate that the stv locus does not ubiquitously respond to thermal stress but is expressed as three distinct thermal and temporal RNA phenotypes (isoforms). The shorter transcript isoforms are rapidly upregulated under stress in all populations and show similar molecular signatures to heat-shock proteins. Multiple stress exposures seem to generate a reserve of pre-mRNAs, effectively ‘priming’ the cells for subsequent stress. Remarkably, we demonstrate a bypass in the splicing blockade in these isoforms, suggesting an essential role for these transcripts under heat stress. Temporal profiles for the weakly heat responsive stv isoform subset show opposing patterns in the two most divergent populations. Innate and induced transcriptome responses to hyperthermia are complex, and warrant moving beyond gene-level analyses.

Keywords: alternative transcript isoforms, Drosophila, stv, thermotolerance

Introduction

Temperature impacts species’ abundance, distribution and susceptibility to environmental change, and is in a phase of unprecedented rise (Hoffmann & Sgro 2011; IPCC 2013). Increasing temperatures are projected to impose significant selection pressures on both endotherms and ectotherms, and there is growing interest in understanding the extent to which organisms will be able to modify upper thermal limits via evolutionary adaptation and mitigate the risk posed by climatic change (Frankham 2005; Hoffmann & Sgro 2011). While many studies have focussed on upper thermal limits at the whole organism level (e.g. Diamond et al. 2012; Kellermann et al. 2012), we still know very little about the link between organismal thermostorances and the cellular processes that underpin their evolution. This is surprising given that the heat-shock response is the most ubiquitous and well-studied stress response (Lindquist & Craig 1988; Yost et al. 1990). At the cellular level, heat shock induces the immediate turnover of molecular chaperones known as the heat-shock proteins (Hsps) which aggregate to protect proteins and partially synthesized peptides through conformational folding and aid in transmembrane transport by stabilizing proteins in a partially folded state (Lindquist & Craig 1988; Kim et al. 2013). The cellular mechanics of heat shock...
are so well characterized that the system serves as a model of gene transcription generally, and with respect to thermal stress. The regulation of the Hsp70 family of chaperones (Guertin et al. 2010) serves as a model in this regard because of its central role in the cellular response to stress. Intensive research of the Drosophila Hsp70s has demonstrated that gene expression is mediated by modulating key steps in the transcription cycle of RNA polymerase II (Pol II), a core component of the mRNA transcribing machinery (comprehensively reviewed in Guertin et al. 2010; Adelman & Lis 2012).

Many fundamental gene classes, including rapidly stress responsive genes, maintain 5′ promoter-proximal enrichment of Pol II, which is engaged but ‘paused’ under nonstressful conditions. Under heat shock, Pol II is released from the pause to undergo elongation by recruitment of the serine/threonine kinase P-TEFb, a process induced by the binding of a specialized transcription factor the ‘master regulator’, heat shock factor (HSF), to target sites harbouring HS sequence elements (HSEs; Birch-Machin et al. 2005; Guertin & Lis 2010; Gonsalves et al. 2011; Teves & Henikoff 2011). The consensous HSE comprises an array of three 5-mer sites in tandem. Under heat stress, HSF trimerizes and binds to bind to HSE’s as a trimer (Perisic et al. 1989), which affects chromatin structure, allowing the recruitment of essential components of the transcriptional protein complex (Guertin et al. 2010).

The thermal activation of Hsp70 is therefore dependent on interactions with a range of cofactors including co-chaperones, the combination of which forms the functional chaperone complex (Arndt et al. 2007). In mammals, the Hsp70-family co-chaperones comprise the Bcl1-associated (BAG) domain proteins, a complex protein family involved in broad processes such as cell cycle and survival, signalling and gene expression (Doong et al. 2002; Coulson et al. 2005; Bonke et al. 2013). The BAG domain, a conserved region of about 50 amino acids near the C-terminal, binds to the ATPase domain of HSP70 to stimulate nucleotide exchange during the ATPase cycle directly regulating HSP70/HSC70 activity (Coulson et al. 2005; Arndt et al. 2007).

Importantly, while the necessity of Hsp70 to mitigate cellular heat shock is unequivocal, its role in underpinning organismal thermotolerance is less well resolved. Attempts to directly link Hsp70 to differences in upper thermal limits have proven difficult. For example, natural variation in HSP70 protein expression was positively correlated with larval thermotolerance in Drosophila (Krebs et al. 1998), and overexpressing HSP70 in transgenic lines also increased larval survival under heat stress (Krebs & Feder 1998; Bettencourt et al. 2008). In adult Drosophila melanogaster, however, marginal or non-significant associations between HSP70 and thermal tolerance have been shown (Dahlgaard et al. 1998; Jensen et al. 2010). By contrast, HSP70 levels showed correlated changes in Drosophila buzzatii lines selected for increased heat tolerance (Sorensen et al. 1999), where thermostolerant Drosophila subobscura strains harbouring ‘warm climate’ inversion polymorphisms showed higher levels of basal HSP70 protein than their cold adapted counterparts bearing the ‘cold climate’ inversion (Calabria et al. 2012). Some of the discrepancy may lie in the stage and/or species specificity of Hsp70 expression, and/or different thermal regimes tested. Fitness costs imposed by heat shock may also impose a trade-off limiting expression (see Calabria et al. 2012). However, it is also likely some inconsistencies may stem from the fact that other aspects of the thermally induced HSP70 complex play an important role in determining differences in upper thermal limits but have so far been largely ignored.

One such emerging candidate is starvin (stv), recently identified as the sole Drosophila BAG protein (Coulson et al. 2005). Stv responds transcriptionally to an array of stressors including cold recovery (Moribe et al. 2001; Colinet & Hoffmann 2010), heat stress (Sorensen et al. 2005; Telonis-Scott et al. 2013) and inbreeding in cold sensitive lines (Vermeulen et al. 2013). Interestingly, stv expression increased as cold tolerance declined with age (Colinet et al. 2013) and showed an interaction between inbred lines and cold stress, suggesting both stage and genotype specificity. While the mode of regulation during cold recovery has not been established, stv, like Hsp70 is regulated by HSF under heat stress (Birch-Machin et al. 2005; Jensen et al. 2008; Guertin & Lis 2010; Gonsalves et al. 2011).

While stv appears rapidly heat inducible, unlike the intron-less Hsps, stv is a complex locus coding seven transcripts and five proteins derived from combinations of alternative transcription and alternative splicing including intron retention (McQuillon et al. 2012). Mechanisms such as alternative transcription and splicing expand transcriptome and proteome diversity through enhanced combinatorial output from a limited range of loci, often increasing phenotypic variation in response to environmental cues such as thermal stress (Faustino & Cooper 2003; Ali & Reddy 2008; Marden 2008; Nilsen & Graveley 2010; Mastrangelo et al. 2012). Given the homology to the human HSP70 BAG co-chaperone (Pagliuca et al. 2003; Coulson et al. 2005) and higher potential for molecular plasticity compared with Hsp70, stv is an intriguing candidate gene that might help better explain variation in upper thermal limits.

Importantly, most stv research to date in the context of thermal stress has ignored this molecular complexity, either focusing on total transcriptional output (Sorensen et al. 2005; Colinet et al. 2013; Vermeulen et al. 2013) or
on the 69KDa MW (stv-PE predicted) protein isoform (i.e., Colinet & Hoffmann 2010). Telonis-Scott et al. (2013), however, demonstrated that stv transcript isoforms are modulated in markedly different ways in response to heat stress, whereby the shorter isoforms underpinned the strong transcriptional response following heat shock. Interestingly, at least a subset of the shorter isoforms showed weak evidence of RNA processing during heat shock which is unusual given that hyperthermia largely inhibits pre-mRNA splicing, a process bypassed in the majority of intron-lacking Hsps (Yost & Lindquist 1986; Bond 1988; Lindquist & Craig 1988). The blockade has been shown to be incomplete in human Hsps with introns (Jolly et al. 1999), but complete in Drosophila Hsp83 (Lindquist 1980; Yost & Lindquist 1986; Corell & Gross 1992).

It is still unclear, however, how stv is linked to thermo-tolerance either geographically and/or under different thermal treatments. Here, we address this by utilizing the natural climatic diversity of the Australian Eastern seaboard where numerous clines have been demonstrated in D. melanogaster at both the trait and gene level (Hoffmann & Weeks 2007). Through common garden experiments on a tropical, mid- and high-latitude population recently derived from nature, we demonstrate genetic (‘basal tolerance’) but not plastic (‘hardened’ tolerance) through prestress differences in knockdown thermotolerance. Using real-time PCR over the two thermal regimes and across a stress/recovery time-course, we show that isoforms of the stv locus do not ubiquitously respond to thermal stress. Rather, they are expressed as three distinct thermal and temporal phenotypes. We observe geographic (population)-specific temporal profiles for the largest and least heat responsive isoform subset, while the temporal profiles of the highly heat-inducible isoforms are mostly conserved across populations, although abundances differ among the populations. Remarkably, we demonstrate for the first time an across-population bypass in the splicing blockade, suggesting an essential role for these transcripts under heat stress. The mode of heat inducibility resulting in differential isoform preference during hyperthermia is discussed.

Materials and methods

Drosophila melanogaster populations and culture conditions

Drosophila melanogaster populations were sampled between February and March 2012 from three locations along the Australian east coast representing ‘high’, ‘mid’ and ‘low’ latitudes; Melbourne (37.99°S, 145.27°E), Port Macquarie (30.93°S, 152.90°E) and Townsville (19.26°S, 146.79°E), respectively. Mass-bred experimental populations were established from 20 (Townsville and Melbourne) or 30 (Port Macquarie) isofemale lines using field caught females. From the isofemale lines from each location, 10 virgin mating pairs were pooled in groups of 400–480 flies, hereafter considered the founding mass-bred generation F0. The populations underwent tetracycline treatment to eliminate potential endosymbionts (Wolbachia) that may cause reproductive incompatibility (Werren 1997). Densities were controlled by randomly mixing 2-day-old flies into fresh potato dextrose medium in 250-mL bottles allowing a standard oviposition window of 4 h. All populations were maintained at >400 flies per generation at 25 °C under a 12:12 h light: dark cycle.

Heat hardening and knockdown assays

We used a static heat knockdown assay to examine innate and plastic thermotolerance (Hoffmann et al. 2002). While the question of how best to study upper thermal limits has been the focus of recent debate, we have shown that this measure provides consistent insight into the adaptive capacity of upper thermal limits in Drosophila (van Heerwaarden & Sgro 2013; Blackburn et al. 2014). Assays were conducted on 6-day-old generation F3 mass-bred females. Imagines were collected into mixed-sex cohorts until 24-h prior to the assays where females were aspirated into groups of 20 without CO2. Flies from each population were randomly assigned into two test groups: (i) ‘basal’ (genetic) thermotolerance or (ii) ‘hardened’ (plastic) thermotolerance. Prior to the knockdown assays, flies allocated to the ‘hardening’ group were subjected to a nonlethal pretreatment (Sgro et al. 2010). Briefly, five groups of 20 flies in 10 dram narrow vials were immersed in a 37 °C water bath for one hour, followed by a 6-h recovery period prior to the knockdown assay. Flies were kept on media throughout hardening and recovery. The untreated ‘basal’ flies were maintained in groups of 20 on media at 25 °C at all times.

For the subsequent static heat knockdown assays, individual females were aspirated into 5-mL dry vials and immersed in a water bath heated to 38.5 °C (following Telonis-Scott et al. 2013), and knockdown time was scored as the time taken to the nearest second for flies to become incapacitated. The knockdown data were generated from three complete blocks of ~35 flies totalling at least 100 individuals per treatment/population.

Quantification of transcript abundance during heat stress

Heat stress sampling. Static heat stress (38.5 °C) was also used to profile the impact of hyperthermia on stv isoform expression during stress and in recovery.
However, unlike the phenotyping assays where files were stressed at 38.5 °C until complete knockdown (from which some flies do not recover), the flies for the transcript assays were subjected to a partial knockdown to ensure that stress-induced transcript expression was not confounded with apoptosis. Further, we deemed it more ecologically relevant to profile flies under extreme stress from which they can recover and survive to reproduce, given that static measures of heat knockdown have been linked to fitness in response to extreme temperature under field conditions (Kristensen et al. 2007). To this end, the time-course was determined by assessing mortality rates 48 h after exposure to increasing increments of heat stress (i.e. 5, 10, 15 min and onwards exposure to heat stress). Flies were subjected to a maximum of 31.5 min at 38.5 °C, after which mortality occurred.

For the transcript expression assays, density was standardized by placing 50 generation F5 eggs into vials. The flies were collected into mixed-sex cohorts until 24-h prior to the assays (at day 5) where females were aspirated into groups of 20 without CO2. For the pretreatment, flies were allocated into either the ‘hardening’ or ‘basal’ groups and treated as described for the heat knockdown assays. For the time series sampling, groups of 20 females were placed in 15-mL Bunzel cryotubes, sampled and snap frozen in liquid N2 according to the following treatments: immediately prestress (25°, on media); during the heat exposure (38.5°, no media) at minutes 15 and 31.5 (referred to herein as 0.25 and 0.53 h, respectively); during the recovery period (25°, on media) at hours 4, 8, 12, 24 and 48 post-exposure (Fig. 1). To control for effects of circadian rhythm on transcription, unstressed flies were also sampled at 12, 24 and 48 h. Three replicates of 20 flies were sampled at each time point for each of the two treatment groups (basal and hardened) across three populations (171 samples).

RNA extraction, cDNA synthesis and real-time PCR. Total RNA was extracted using the mini RNA isolation kit (Bioline) and DNase treated using the TURBO DNA-free™ kit (Ambion) to remove residual genomic DNA. The purified RNA was quantified on a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and integrity assessed visually via 1% agarose gel electrophoresis.

Complementary DNA was synthesized from 500 ng of RNA in a 20-μL volume. The reverse transcription reaction was performed using 4 μL 2.5 mM dNTPs, 2 μL 40 μM oligo-dT primer, and DEPC water. The mixture was incubated at 70 °C for 5 min then cooled on ice before adding 2 μL 10× RT buffer and 1 μL of M-MuLV reverse transcriptase (200 U/μL). The samples were incubated at 42 °C for 1 h, followed by enzyme deactivation at 90 °C for 10 min. The cDNA was diluted 1:10 in water. Real-time PCRs (10 μL) were performed in 384-well plate format using a Roche LightCycler® 480 and SYBR® Green chemistry. Transcripts were amplified using LightCycler® 480 SYBR Green I master-mix. Each well contained 5 μL PCR buffer, 4 μL 1 μM primer mix and 1 μL diluted cDNA. Reactions were performed in duplicate for each cDNA sample, with three biological replicates for each population/treatment/time point combination. All populations and transcripts corresponding to the same gene/treatment were run on the

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Fig. 1 Treatment and sampling schematic for the two thermal regimes, nonlethal hardening treatment (dashed box) and subsequent severe thermal stress (solid box). Prior to severe thermal stress, groups of 20 female flies either underwent 1-h exposure to 37 °C followed by 6-h recovery at 25 °C or were maintained constantly at 25 °C. Time zero represents 7 h following hardening or constant temperature treatments. Flies were sampled immediately prestress (time zero), 0.25 and 0.53 h at 38.5 °C. Flies sampled during recovery were exposed to 0.53 h at 38.5 °C, which represented population upper thermal limits without ensuing mortality. Crosses indicate sampling time points where flies were snap-frozen for the transcript expression analysis. Note, time is not drawn to scale.

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same plate with biological replicates run on separate plates.

Transcript primer sequences were designed using PRIMER-BLAST (NCBI), QUANTPRIME and GETPRIME (Arvidsson et al. 2008; Gubelmann et al. 2011) (Table 1, Fig. 2). Owing to overlapping low sequence complexity at the long stv:RA:RE:RF and mid RB:RC:RG exon junctions, primers were designed to amplify the transcript subsets, while RD was amplified individually (see gene schematic, Fig. 2). Primers were designed to detect both mature stv transcripts from the alternative start exon junctions and the pre-mRNA from exon/intron primers at the first exon (Fig. 2). Transcript/subset abundance was calculated relative to the thermally and temporally stable ‘housekeeping’ gene RPL11 (Telonis-Scott et al. 2013), where relative expression of transcript of interest (TOI) = 2(ΔC(TOT11 – TOI)). Thermo-stability of RPL11 was verified in the populations using a one-way ANOVA with the fixed effect of time point. As we had a priori expression information from microarray data (Telonis-Scott et al. 2013), RPL11 was considered a sufficiently stable as a reference ‘control’ gene. Expression patterns were verified in the population real-time PCR data.

Statistical analyses

Genetic and plastic measures of thermotolerance. The effects of latitude and thermal regime were examined using two-way mixed-model analysis of variance (ANOVA) with population and treatment (basal tolerance or hardening response) as fixed factors, run as a random factor and the interaction between population and treatment. Residual diagnostics were performed using (PROC UNIVARIATE, SAS v9.3), and while the data were predominantly normally distributed, the diagnostics indicated a slight departure from normality (Shapiro–Wilk test, P < 0.05). Several models were fit to better account for this including fitting a separate model for basal and hardening, a mixed linear model with run (block) as a random factor, and a generalized linear model, both on log transformed and untransformed data. The best fit was a mixed model on untransformed data invoking the REPEATED/SUBJECT = replicate (population, time

Table 1 Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcript/Subset</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
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<td>RPL11</td>
<td>RA:RB</td>
<td>CGATCCCTCCATCGGTATCT</td>
<td>AACCACCTCATGGCCTCT</td>
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<tr>
<td>stv</td>
<td>Pre-RA:RE:RF</td>
<td>CCAAAAGCCCTTACCGATCG</td>
<td>GGGGGGCACTGAACCTGAA</td>
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<tr>
<td></td>
<td>Pre-RG:RB:RC</td>
<td>AAGCGGAAAAAGCATTCAA</td>
<td>GATGTCGATGTCGGAACCTT</td>
</tr>
<tr>
<td></td>
<td>RA:RE:RF</td>
<td>CACAGTTCACACTCCCCA</td>
<td>GAATCAGGGACTGGGCTGAA</td>
</tr>
<tr>
<td>RB:RC:RG</td>
<td></td>
<td>GTCCACAAACGGGAAAACAT</td>
<td>CAAAGGTCGCGGTTGTGGCCT</td>
</tr>
<tr>
<td>RD</td>
<td>ACATAGTGTATGTGAAACAGCG</td>
<td>CCAAGGTCGCGGCTTTTATATT</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 stv gene model showing the gene region (chromosome 3L:13470641-13476615), long RA:RE:RF isoform subset, short isoform subset RB:RC:RG and smallest RD isoform each derived by alternative start exons. The lines joining exon junctions indicate primer sites for processed (alternatively transcribed and spliced) transcripts targeting the alternative start exons and the line in the first exon/intron indicates the primer pair used to amplify the primary stv pre-mRNA. The white boxes indicate 5’ and 3’ UTRS, while the black boxes show the coding regions. Schematic adapted from Flybase V2014_02 (McQuilton et al. 2012).
point) and the GROUP statements = treatment (basal or hardened) (PROC MIXED, SAS v9.3) to account for the different basal and hardening variances.

**Transcriptional responses to thermal stress.** The effect of thermal regime and population on the temporal expression of the *stv* isoform/subsets was analysed using ANOVAS and planned contrasts. All transcripts were log transformed for linearity and were initially assessed with a four-way fixed effects linear model fitting transcript, treatment, time point and population and interaction terms (PROC GLM, SAS v9.3). Residual diagnostics, however, revealed strong heteroskedasticity driven by differences in variances between the treatments and *stv* isoforms (i.e. non-normality of the hardened residuals). To better fit the different variances, mixed-model ANOVAS with the fixed effects of population, treatment, time point and interaction terms were applied where REPEATED/SUBJECT = replicate (population, time point) and the GROUP statement = treatment (basal or hardened) (PROC MIXED, SAS v9.3). As for the heat knockdown data, multiple models were examined; however, the mixed linear model using the GROUP and REPEATED statements better accounted for the different basal and hardening variances (no random term was fit). Initially, a full model including transcripts was fit for *stv*, but as the different transcripts presented as separate phenotypes, a separate model was fit for each isoform set to more subtly detect the effects of treatment, time point and population. Reduced models were fit and are presented where the higher order interaction term was nonsignificant. The impact of circadian rhythm did not impact transcript expression (nor differ from prestress), and the unstressed time points at 12, 24 and 48 h were excluded from the final analyses.

Where there was a significant time-by-treatment interaction term, planned contrasts were performed to more finely dissect differences in temporal profiles. Contrasts were deemed more informative within treatments and between populations given the strong treatment effect and general lack of overall significant population term for most transcripts. Relative expression to time zero (prestress) was compared as well as absolute expression between time points. For the long transcript isoforms *stv*-RA:RE:RF and middle isoforms *stv*-RG:RB:RC, a total of 56 contrasts were run for each population, and 12 tests were run for the short isoform *stv*-D. Relative levels of basal vs. hardened *stv*-RA:RE:RF, pre-RD:RE:RF, *stv*-RG:RB:RC and pre-RG:RB:RC were compared, respectively, by population, for a total of eight comparisons each. P-values were corrected for multiple tests using a false discovery rate (FDR) approach (Benjamini & Hochberg 1995). 

**Results**

*Genetic and plastic differences in heat knockdown time*

For average knockdown time, two-way ANOVA showed significant differences among populations as well as a strong treatment effect; however, there was no interaction between population and treatment (Table 2, Fig. 3). Planned contrasts by treatment showed that the tropical low-latitude population (Townsville) had higher average basal knockdown resistance than the mid (Port Macquarie) and high-latitude (Melbourne) populations (high latitude vs. low latitude, *F* = 11.19, d.f. = 1, *P* < 0.001; mid latitude vs. low latitude, *F* = 3.97, d.f. = 1, *P* < 0.05). The mid- and high-latitude populations did not differ from each other for basal average knockdown. There were no differences among the three populations for hardened knockdown time, which improved tolerance on average 14 min (Fig. 3).

***Stv* ANOVA**

Three-way ANOVA were fit for each transcript/subset separately. The overall effect of population and time point were significant for the longest isoform subset *RA:RE:RF* (for gene model see Fig. 2), but there was no significant effect of thermal regime (basal or hardened treatments) and no interaction between effects (Table 3). By contrast, the middle isoforms *RG:RB:RC* showed a marginal effect of treatment, and a strong effect of time point and a significant time point-by-treatment interaction term (Table 3). For the time points discernable for the shortest isoform *RD* (4- to 24-h recovery), there were highly significant treatment and time point terms, treatment-by-time point interaction and marginal treatment-by-population interaction (Table 3).

**Temporal, thermal regime and geographic variation of *stv* isoforms**

Given that we identified major treatment-by-time interactions, we utilized planned contrasts to better dissect

### Table 2 Two-way mixed-model ANOVA results showing the fixed effects of population and treatment (basal or hardened), and the interaction term for heat knockdown

<table>
<thead>
<tr>
<th>Main effects</th>
<th>d.f.</th>
<th><em>F</em>-value</th>
<th><em>P</em>-value</th>
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<td>Population</td>
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<td>5.64</td>
<td>0.0038</td>
</tr>
<tr>
<td>Treatment*population</td>
<td>2</td>
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<td>0.1856</td>
</tr>
<tr>
<td>Error</td>
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</table>

Significant terms are bolded.
patterns between time points, where absolute expression and expression relative to prestress (fold induction) were compared by population. Given the large number of factors and levels within (i.e. time points) ANOVA alone was not sufficiently powerful to explore obvious variations in temporal profiles, hence, we included the long isoform subset (RA:RE:RF) expression in this analysis despite a lack of interaction term.

Overall, three distinctive thermal expression phenotypes were revealed. Notably, the long isoforms were weakly inducible during recovery, lacked marked expression differences between basal and hardened treatments across the time series, but exhibited population-specific profiles for the two thermal regimes during recovery. By contrast, the middle isoforms were rapidly heat inducible in high abundance and showed different temporal profiles for the treatments that were consistent among the populations. While the latter isoforms are constitutively expressed under nonstress conditions, the shortest isoform was only induced by heat shock, with consistent expression captured by 4-h recovery. It is likely, however, that levels of this isoform accumulated during thermal stress, as some signal was observed during this period, but abundances were not consistently within a reliable detection threshold using relative real-time PCR and were therefore excluded from the analyses. By 48-h recovery, RD transcripts were no longer detectable.

‘Long’ isoforms: stv-RA:RE:RF. Contrasts of absolute expression variation between time points by population showed that the longest stv transcripts were not elevated until 4-h recovery regardless of thermal regime or latitude of origin. However, population-specific differences were observed between basal and hardened expression profiles between 4- and 8-h recovery. Interestingly, the most phenotypically divergent populations mounted different expression responses during stress

Table 3 Three-way mixed-model ANOVA results for the fixed effects of treatment (basal or hardened), time point and population for the mature isoform/subsets of stv during and in recovery from severe thermal stress

<table>
<thead>
<tr>
<th>Main effects</th>
<th>d.f.</th>
<th>F-value</th>
<th>P-value</th>
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<tr>
<td>Treatment*time</td>
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<tr>
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<td>Stv-RG:RB:RC</td>
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</table>

Significant terms are bolded.
and recovery according to treatment. In the basal treatment, the high-latitude flies expressed peak RA:RE: RF transcripts at 4- and 8-h recovery (0.25 vs. 4 h: 0.53 vs. 4 h: FDR corrected \( P < 0.001 \), 0.25 vs. 8 h, 0.53 vs. 8 h: FDR corrected \( P < 0.05 \), Fig. 4A, Table S1, Supporting information), with no significant change in hardened profiles. By contrast, the low-latitude flies expressed more RA:RE:RF at 4-h recovery following the hardening treatment (0.25 vs. 4 h, 0.53 vs. 4 h, 4 vs. 8 h: FDR corrected \( P < 0.001 \), 4 vs. 12 h: FDR corrected \( P < 0.01 \), Fig. 4A, Table S1, Supporting information). The mid-latitude population exhibited a high/low ‘intermediate’ profile, with significant peaks compared to stress at 4- and 8-h recovery in unhardened flies (0.53 vs. 4 h, 0.53 vs. 8 h, FDR corrected \( P < 0.05 \), but with an additional peak at 4-h recovery following hardening (0.25 vs. 4 h, 0.53 vs. 4 h, FDR corrected \( P < 0.05 \), Fig. 4A, Table S1, Supporting information).

Comparing fold-changes across the time series and treatments reflected the lack of early thermal inducibility of the long transcripts. This was consistent across both treatments where pretreatment for an hour at 37 °C did not impact prestress transcripts prior to exposure to 38.5 °C. Levels of significant upregulation during the recovery period compared to prestress were also slight, where the high and mid populations peaked at 3.7- and 3-fold, respectively, at 4-h recovery (FDR corrected, \( P < 0.01 \), Fig. 5A, Table S1, Supporting information), remaining similar at 2.9-fold at 8-h recovery (FDR corrected \( P < 0.05 \), and 0.01, Fig. 5A, Table S1, Supporting information). The tropical population exhibited the largest shift after hardening at 4-h recovery, upregulated on average fivefold compared to prestress, while unhardened flies exhibited a later shift with peak induction of almost threefold at 8 h (FDR corrected \( P < 0.0001 \) and \( P < 0.05 \), respectively, Fig. 5A Table S1, Supporting information).

‘Middle’ isoforms: stv-RG:RB:RC. Planned contrasts by population across the time points and treatments revealed many significant comparisons, although the patterns were largely similar across populations (Table S1, Supporting information). Notably in all populations for the basal treatment, the mid-isoform subset (RG:RB:RC) was induced during stress and peaked at 4-h recovery in (0.25 vs. 4 h: FDR corrected \( P < 0.0001 \); 0.53 vs. 4 h: FDR corrected \( P < 0.0001 \), Fig. 4B, Table S1, Supporting information). Despite transcript levels declining significantly by 8-h recovery and into later recovery, expression was maintained from 8-h recovery at higher levels than prestress, before returning to prestress levels by 48-h recovery (Fig. 4B, Table S1, Supporting information).

The hardening treatment impacted transcription of the middle subset resulting in greater accumulation of transcripts at prestress levels and attenuated expression compared to unhardened flies and a temporal shift to peak expression during stress (Fig. 4B, Table S1, Supporting information).

Planned contrasts relative to prestress showed that basal levels were significantly upregulated (0 vs. all time points, all populations, FDR corrected \( P < 0.0001 \), Fig. 5B, Table S1, Supporting information). Fold changes were consistently between 5- and 10-fold higher than prestress, peaking on average around 80-fold by 4-h recovery, and remained at least on average 30-fold higher at 24-h recovery (Fig. 5B, Table S1, Supporting information).

Hardening resulted in slight differential regulation of the mid-isoforms relative to prestress. Although only the high- and low-latitude populations were statistically significant in the planned comparisons, trends were similar for mid-latitude flies, which tended towards overall broader expression variances (low latitude: 0 vs. 0.25 h, 0 vs. 0.53 h FDR corrected \( P < 0.05 \), high latitude, 0 vs. 0.53, FDR corrected \( P < 0.01 \), Fig. 5B, Table S1, Supporting information). Fold changes indicated upregulation on average of twofold during stress, although transcripts were significantly downregulated compared to prestress levels at 24- and 48-h recovery in the low- and high-latitude populations (0 vs. 24 h, FDR corrected \( P < 0.05 \), 0 vs. 48 h, \( P < 0.05 \), Melbourne \( P < 0.01 \), respectively, Fig. 5B, Table S1, Supporting information).

‘Short’ isoform: stv-RD. As levels of the heat-inducible isoform were undetectable prestress, we were restricted to comparisons from 4-h recovery. While absolute expression levels were of an order of magnitude lower than the other stv isoforms (Fig. 4A), both the basal and hardening treatments elicited transcription. Like the mid-isoforms (stv-RG:RB:RC), basal expression was highest at 4-h recovery while expression was significantly reduced following hardening (Fig. 4C). Unlike the latter transcripts, however, high levels of the shortest stv isoform were not maintained after 4-h recovery, albeit levels remained detectable until 24-h recovery, suggesting that the transcripts were still above prestress levels until this time (4 vs. 8 h, high- and mid-latitude populations FDR corrected \( P < 0.05 \) and 0.001, respectively, 4 vs. 12 h, \( P < 0.001 \), 4 vs. 24 h, \( P < 0.0001 \) Fig. 4C, Table S1, Supporting information). The very slight treatment-by-population effect was likely due to expression variation specific to hardened tropical flies, where there were significant differences between 8 and 12 h, and 8 and 24-h expression (FDR corrected \( P < 0.05 \) and 0.01, respectively, Table S1, Supporting information).
Hardening maintained high pre-RG:RB:RC (‘middle’ isoforms) levels well after recovery from subsequent stress

Evidence in whole animals exploring expression of primary transcripts during hyperthermia demonstrated that transcription occurs as primary transcripts accumulate with increasing temperature, while mature transcripts decline considerably over 35°C congruent with the splicing block in an inbred *Drosophila melanogaster* strain (T. K. Johnson, PhD Thesis 2010, Monash University, Australia, unpublished data) as well as an outbred strain tested at 38.5°C (Telonis-Scott et al. 2013). Here, we examined expression of the primary long and mid *stv* isoforms as a proxy for transcription rates during stress and recovery to see how they track with the mature transcripts and to determine the impact of the hardening exposure.

As one primer each for pre-RA:RE:RF (long isoform subset) and pre-RG:RB:RC (middle subset) was designed in intronic sequence (Fig. 2), it was anticipated that the middle isoforms precursor abundances would also comprise the long isoform precursor abundances. However, this issue was negligible due to the order of magnitude higher abundance of pre-RG:RB:RC compared to pre-RA:RE:RF, where expression patterns remained stable following subtraction of pre-RA:RE:RF transcripts (data not shown).

Fig. 4 Geographic, temporal and isoform variation in expression of *stv* under two different thermal regimes. All isoform/subsets are shown relative to *RPL11*, dashed lines = heat shock at 38.5°C, solid lines = recovery at 25°C after exposure for 31.5 min. Black lines = unhardened expression, grey lines = expression following hardening at 37°C for 1 h prestress. (A) The weakly inducible long isoform subset (RA:RE:RF) was expressed in population-specific manner where the extreme latitude populations showed opposite expression patterns for the basal and hardened treatments at 4- and 8-h recovery, while the mid-latitude population exhibited an intermediate profile. (B) The stress-inducible mid-isoform subset (RG:RB:RC) isoform subset was upregulated during stress with peak expression at 4-h recovery that was one and two orders of magnitude higher than the other isoforms. Hardening resulted higher pre-stress levels and the temporal shift in peak expression to stress. (C) Expression of the shortest heat shock-specific RD isoform is only presented from 4-h recovery where quantification was reliable. Similar to RG:RB:RC, expression peaked at 4 h, although this transcript was undetectable by 48-h recovery. Hardening impacted RD during recovery similarly to RG:RB:RC. Error bars represent ± SE of the mean.
ANOVA on pre-RA:RE:RF levels was significant for treatment, time point, and the interaction term, and the population differences observed in the mature transcripts were reflected in the significant treatment-by-population term (Table 4). These results suggest the potentially greater sensitivity of pre-RA:RF:RE transcripts as a measure of transcription rates where the geographic variation in expression patterns according to treatment observed in the mature transcripts is more evident in the primary RNA. The average effect of thermal treatment was greater on unprocessed RG:RB:RC transcripts compared to mature transcripts, with a highly significant treatment term in the ANOVA (Table 4).

From visual comparison of the primary and mature transcripts over the time series, it was evident that for the middle isoform subset, the impact of hardening was different depending on the maturity of the transcript. To further explore this, we used planned contrasts to dissect the time point-by-treatment interaction but focussed on direct comparisons of the two treatments (basal vs. hardened) rather than contrasting time points to each other as for the temporal profiling above. This was carried out for both the primary and mature RA:RE:RF and RG:RB:RC isoforms subsets separately.

Primary levels of the long stv isoforms were extremely low and tended to track the mature transcripts which remained in a steady state during stress with only weak inducement during recovery, that is the pre-mRNAs increased negligibly during stress consistent with only a small rise necessary to maintain the transcript pool. When the populations were compared separately, there were virtually no differences between basal and hardened expression at each time point apart from a rise in hardened pre-mRNAs compared to basal after 15 min of stress in the high-latitude population (Fig. 6A, FDR corrected $P < 0.05$, Table S2, Supporting information). This was also reflected in the mature transcripts, where expression in hardened vs. basal flies was highest in the low-latitude population prestress and after 15 min of stress (Fig. 6A, FDR corrected $P < 0.05$, Table S2, Supporting information). The population differences during the treatments in recovery were also reflected where following hardening, the tropical (low latitude) population expressed much more...
higher mature RA:RF:RE transcripts at 4-h recovery than basal flies and compared to the lower latitude populations (Fig. 6A, FDR corrected \( P < 0.05 \), Table S2, Supporting information).

Compared to the long isoforms, pre-RG:RB:RC levels were considerably higher regardless of treatment, although hardening further increased both primary and mature transcripts prior to the severe stress at 38.5 °C (all populations pre-RG:RB:RC and mature RG:RB:RC basal vs. hardened 0 h FDR corrected \( P \)-value < 0.0001, Fig. 6B, Table S2, Supporting information). Mature transcripts expressed after hardening showed the temporal shift to peak expression that was higher than basal levels during stress (all populations, RG:RB:RC basal vs. hardened 0.25 h, Fig. 6B, Table S2, Supporting information). The three HSEs are positioned within the presumed regulatory region for the mid and short isoforms (stv-RG:RB:RC and stv-RD) and include one site that rests in close proximity to stv-RG:RB:RC (at -106-92) which has been previously identified to bind HSF (Gonsalves et al. 2011). The HSE located furthest upstream closely resembles this site and is positioned in the centre of the 5' untranslated region for the stv-RA:RE:RF isoforms.

**Discussion**

Our study represents the first analyses of natural genetic variation for thermotolerance and the molecular complexity of stv across different thermal regimes. We first demonstrated that *Drosophila melanogaster* females from the tropics were more heat tolerant than higher latitude populations providing an excellent system to explore links with *stv* expression and evolved differences in upper thermal limits. While each population represents a single average measurement of the different traits from the three locations (latitudes), multiple genotypes were collected across each site and pooled into a single ‘population’. This approach has successfully been utilized to compare a range of interpopulation phenotypes including gene expression, and has identified strong, stable geographic patterns (i.e. Hoffmann et al. 2002; Sgro et al. 2010; Telonis-Scott et al. 2011). Here, the data corroborate previous intrapopula-

*Table 4 Three-way mixed-model ANOVA results for the fixed effects of treatment (basal or hardened), time point and the stv pre-RA:RE:RF (long isoforms) and pre-RG:RB:RC (middle) isoforms, during and in recovery from severe thermal stress*

<table>
<thead>
<tr>
<th>Main effects</th>
<th>d.f.</th>
<th>( F )-value</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stv-RA:RE:RF premRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>24.29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>7</td>
<td>11.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Population</td>
<td>2</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td>Treatment*time</td>
<td>7</td>
<td>2.11</td>
<td>0.04</td>
</tr>
<tr>
<td>Treatment*pop</td>
<td>2</td>
<td>3.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Time*pop</td>
<td>14</td>
<td>0.93</td>
<td>0.52</td>
</tr>
<tr>
<td>Stv-RG:RB:RC pre-mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>51.95</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>7</td>
<td>52.82</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Population</td>
<td>2</td>
<td>1.97</td>
<td>0.14</td>
</tr>
<tr>
<td>Treatment*time</td>
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<td>20.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment*pop</td>
<td>2</td>
<td>1.15</td>
<td>0.32</td>
</tr>
<tr>
<td>Time*pop</td>
<td>14</td>
<td>1.18</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Note planned contrasts were conducted by population within each pre-mRNA subset as for the mature transcripts but also contrasted directly to the mature transcript subset by population and time point. Significant terms are bolded.
tion latitudinal variation observed for heat knockdown along the Australian east coast (Hoffmann et al. 2002; Sgro et al. 2010). Sgro et al. (2010), however, found that hardening capacities tended to increase towards the tropics. In contrast, while we observed strong hardening responses across all populations, we found no differences in phenotypic plasticity for heat knockdown among the populations. This is likely due to our sampling three populations representing low, mid and high latitudes vs. multiple populations spanning the climatic gradient, which affords the most power to detect clinal patterns. In addition, it is worth noting that using the same treatment to induce a hardening (plastic) response in populations that differ in basal thermotolerance raises the questions as to whether we were in fact comparing the same plastic response across populations. We have previously shown (Sgro et al. 2010) that such an empirical approach can still provide insight into adaptive divergence in thermotolerance across populations. Nonetheless, this is an issue that should be addressed in future empirical work.

We next sought to determine whether the differences in thermal phenotypes were reflected at the molecular level in the different *stv* isoforms. Informed by our previous genomewide analyses of transcriptional responses to severe thermal stress (Telonis-Scott et al. 2013), we employed detailed time series analyses to partition the genetic from plastic responses by comparing both basal and hardened flies subjected to the same severe thermal stress.

**Table 5** Identification of putative heat-shock elements (HSEs) in the *stv* regulatory region

<table>
<thead>
<tr>
<th>Putative HSE sequence</th>
<th>Chromosomal location</th>
<th>Location relative to <em>stv</em> TSS (isoforms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGAACATACGAGAAG</td>
<td>3L:13470997..13471011</td>
<td>−2548 to 2534 (RG:RB)</td>
</tr>
<tr>
<td>TGAAAATTTTCTAGAAG*</td>
<td>3L:13472607..13472622</td>
<td>−938 to 923 (RG:RB)</td>
</tr>
<tr>
<td>AGAAACTACGAGAAG*</td>
<td>3L:13473439..13473453</td>
<td>−106 to 92 (RG:RB)</td>
</tr>
</tbody>
</table>

TSS, transcriptional start site.
*Empirical evidence exists for heat shock factor binding (Gonsalves et al. 2011).

Fig. 6 *stv* pre- and mature mRNA isoform expression under two thermal regimes. Transcripts are shown log2 transformed relative to RPL11 to directly contrast primary (dashed lines) and processed (solid lines) mRNA levels. Basal flies are shown in black, hardened are shown in grey. (A) Long isoform subset (RA:RE:RF): significantly lower levels of primary transcripts compared to processed transcripts were observed across the time series and the pre-mRNAs remained mostly in a steady state tracking the low inducement of the mature transcripts in recovery. (B) Middle isoform subset (RG:RB:RC): pre-RG:RB:RC levels where higher consistent with the high inducibility of this transcript set compared to RA:RF:RE, and we found evidence for a longer term molecular hardening response at the RG:RB:RC transcript precursor level that was not apparent in the mature transcripts suggestive that multiple exposures may maintain a reserve of pre-mRNAs.
et al. 2013). Our current results confirm these patterns and, importantly, go further to demonstrate that isoform expression is genotype and treatment specific. stv therefore is an intriguingly complicated locus that encodes essential constitutively expressed products during development and throughout life history (e.g. Coulson et al. 2005; Arndt et al. 2010; Graveley et al. 2011; Eddison et al. 2012), but also rapidly switches to high thermal inducibility from a constitutively expressed isoform set (middle isoforms, RG:RB:RC) and by invoking transcription of a rare isoform expressed usually under few developmental/tissue stages (short isoform, RD) (Graveley et al. 2011). Our survey of potential HSF binding sites in the stv regulatory region revealed three potential HSEs including one previously shown to bind HSF (Guertin & Lis 2010; Gonsalves et al. 2011). Consistent with our observations that stv-RG:RB:RC and stv-RD are highly heat inducible, we find all three sites upstream of the TSS for these isoforms and downstream of the weakly induced long isoforms. The heat-inducible isoforms were expressed in a geographically conserved manner and exhibited an analogous molecular signal to heat shock and hardening to Hsps such as Hsp70 and Hsp68, which are immensely upregulated from basal levels during stress and exhibit maximum expression during early recovery (i.e. Vazquez et al. 1993). These ‘Hsp-like’ patterns are highly stable given that our earlier time series clustering showed that, at the whole gene level, stv and Hsp68 were co-expressed while Hsp70 members were assigned to a different but representative profile (Telonis-Scott et al. 2013). This is likely because the rate and magnitude of Hsp70 expression is greater than other Hsps at both the transcript and protein level (i.e. Lindquist 1980; Vazquez et al. 1993).

Further, hardening at 37 °C for 1 h increased RG:RB:RC levels at the onset of the more severe stress compared to basal flies even with a 6-h recovery, an occurrence that is well documented in Hsps. Pre-accumulation and maintenance of Hsps is thought to at least partly underlie the improvement in thermotolerance following hardening (Lindquist & Craig 1988; Yost et al. 1990; Feder & Hofmann 1999). Here, the plastic molecular response during subsequent severe stress in the heat-inducible isoforms was distinct from basal flies, congruent with Hsp-like induction, but similar among populations, congruent with the low plastic phenotypic variance observed, although refer to above regarding hardening regimes. The ‘double’ stress imposed by hardening treatment plus subsequent stress resulted in a greater reserve of pre-mRNAs after 48 h, which suggests that hardening can be maintained potentially as a longer term response at the transcriptional level, long after processing of the mature transcript has declined.

For the weakly induced long isoforms, while we did not detect HSEs immediately upstream of TSS, the induction of these transcripts at 4-h recovery suggests that a distal HSE may be contributing to their mild increase. Notably, however, the most distant putative HSE is located within the 5’UTR of stv-RA:RE:RF. Given that HSF can act in a repressor capacity, it is tempting to speculate that binding of HSF here might act to repress further transcription of these isoforms during heat shock and instead shift production to that of the shorter messages (Westwood et al. 1991; Chen et al. 2009). Preliminary blasts of proteins encoded by the different isoforms do not detect known domains in the variable regions, suggesting perhaps it is not preference for isoforms with variable functions driving expression of the shorter isoforms, but rather costs imposed by differences in message production under hyperthermia.

Interestingly, the weakly heat-inducible long isoforms showed population-specific expression differences compared to the heat-inducible mid- and short isoforms with HSEs upstream of the TSS. Notably, the most divergent populations for heat tolerance, low latitude (tropical Townsville) and high latitude (Melbourne) showed opposing patterns according to treatment during recovery, while the mid-latitude population exhibited an intermediate profile. This isoform-specific geographic complexity would have been missed in the standard ‘whole’ gene analyses of this locus because of the order of magnitude difference in expression of the isoform subsets. The results reflect the possibility that different elements of the same locus may be under different selection pressures, a process afforded by the plasticity of the transcriptome through mechanisms such as alternative transcription and splice sites (Keren et al. 2010). Further, the divergence of the long isoform expression is highest in the most tropical and thermo-tolerant population (Townsville), suggesting that variation here could be linked to climatic selection. Whether these patterns imply an essential role for the shorter isoforms during heat shock related to the proximity of HSEs, and/or for a different role of the longer isoforms during thermal recovery remains to be tested.

Remarkably, we observed high levels of processed RG:RB:RC transcripts during severe heat stress which apart from isoforms of HSF itself (Fujikake et al. 2005) are one of the first loci shown to bypass the splicing blockade in Drosophila. stv-RG:RB:RC mRNAs are derived from a complex combination of alternative transcription and splicing, including the rarer event in Drosophila of intron retention in the RC isoform (Fig. 2). The RG:RB:RC subset is derived from the alternative start site in the 5’UTR, an exon interrupted by intronic sequence (Fig. 2). As primers were designed across the common exon-junction joining the 5’UTR of the RG:RB:
RC subset, only processed (or at a minimum partially processed given the coupling of transcription and splicing; reviewed in Pal et al. 2012) transcripts would have amplified during the PCRs. Interestingly, the RA:RE:RF subset was expressed in unhardened flies similarly to Hsp83, an intron containing Hsp that is subject to the splicing block until restoration of splicing at less severe temperature in Drosophila cell lines (Lindquist 1980; Yost & Lindquist 1986; Corell & Gross 1992). However, while Hsp83 splicing has been shown to be rescued by hardening (Yost & Lindquist 1986), RA:RE:RF isoforms accumulated in their primary state during heat stress in both treatments. This may at least in part be because of the potential low thermo-inducibility of the RA:RE:RF isoforms at the regulatory level compared to Hsp83.

The outstanding questions remain as to why such complex transcripts can behave similarly to transcripts evolved specifically for rapid turnover during heat shock, and whether the stv transcripts are actually fully translated during heat shock. Based on sequence and protein conservation, stv is a homologue of the human BAG3 gene (Coulson et al. 2005; Colinet & Hoffmann 2010). While the BAGs (i.e. 1, 2 and 5) colocalize with HSP70/90 in the ubiquitin/proteasome system (the main degradation pathway for mis-folded proteins; Arndt et al. 2007) to date BAG-3 is the only stress inducible BAG shown to be coordinately expressed with HSP70 under hyperthermia (Pagliuca et al. 2003; Rosati et al. 2007). The BAG-3 protein was highly expressed with HSP70 in human HeLa cells shocked at 42 °C for 30 min, and concentrations of both mRNAs increased between 30 min and 4 h into stress (Pagliuca et al. 2003). The authors proposed that BAG-3 may modulate the folding activity of Hsc/HSP70 chaperone machinery, plus also influence the anti-apoptotic properties of HSP70 to maintain cell survival under stress. In D. melanogaster, the evidence so far for stv transcript and protein coregulation with HSP70 is restricted to cold stress recovery, but not stress per se (Colinet & Hoffmann 2010).

Both BAG3 and stv encode different isoforms through alternative promoters and contain introns, and therefore, it is intriguing that splicing efficiency is maintained under high heat and that full protein expression is maintained in BAG3 despite the processing complexity required. Few genes so far have been demonstrated to bypass the splicing block, and stv is among the first to be characterized in Drosophila apart from HSF itself. Using real-time PCR and reporter assays, Fujikake et al. (2005) showed that isoforms of HSF are alternatively spliced and fully processed at 37 °C, but did not elaborate on the mechanisms of the bypass. In human cells, Jolly et al. (1999) showed that Hsp HSF sites become associated with splicing factor ‘speckles’ during hyperthermia regardless of intron status, resulting in complete splicing of 10 introns from Hsp90. Dissecting the mechanism of processing protection for stv transcripts presents a new research avenue which may also shed light on the role for products of this gene in thermotolerance.

Conclusion

Stv is a complex locus that produces different transcript and protein isoforms based on environmental cues, encompassing developmental, tissue or genotype specificity, but little is known about this locus under heat shock. Here, we link the molecular complexity of stv isoforms to different thermal challenges in different genetic backgrounds from variable climates. We found that the shorter isoforms are favoured under high heat regardless of genetic background, are fully processed yet show similar molecular signatures to well-known Hsps despite their complexity. We found evidence for a longer term molecular hardening response at the transcript precursor level that was not apparent in the mature transcripts which could imply that multiple exposures may maintain a reserve of pre-mRNAs effectively ‘priming’ the cells for subsequent stress. Interestingly, the longest and most weakly, induced isoform subset proved to be most variable among the populations from different latitudes, such that the most divergent populations showed opposite molecular signatures. Whether these patterns imply an essential role for the shorter isoforms during heat shock related to the more proximal HSEs compared to the longer isoform with more distal HSEs remains to be tested.

Outstanding questions remain as to whether stv protein isoforms are processed and transported under high heat and how they interact with HSP70. Further, population genetic analyses around this locus could address whether there are patterns of sequence divergence that relate to the different expression variation, and if ultimately they are associated with evolved differences in thermotolerances. We suggest that the same isoforms revealed here likely underpin the high heat-shock gene expression observed in other studies and highlight the importance of considering genes in their complexity, not just as a single transcriptional unit as assessed by many researchers studying stress responses.

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References


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Data accessibility
The thermal phenotype and real-time PCR data are available from https://datadryad.org/ doi: 10.5061/dryad.63t28.

Supporting information
Additional supporting information may be found in the online version of this article.

Table S1. Planned contrasts with FDR corrections for all mature transcripts.

Table S2. Planned contrasts with FDR corrections for primary transcripts.