MOLECULAR ECOLOGY

Molecular Ecology (2014) **23**, 6135–6151 doi: 10.1111/mec.13000

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

MARINA TELONIS-SCOTT,* ALLANNAH S. CLEMSON,* TRAVIS K. JOHNSON*† and CARLA M. SGRÒ*

*School of Biological Sciences, Monash University, Clayton, Vic. 3800, Australia, †Department of Biochemistry and Molecular Biology, Monash University, Clayton, Vic. 3800, Australia

## 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

*Received 7 September 2014; revision received 6 November 2014; accepted 13 November 2014*

## 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 thermotolerances 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

Correspondence: Marina Telonis-Scott, Fax: 613 9905 5613; E-mail: marina.telonisscott@monash.edu

© 2014 John Wiley & Sons Ltd

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 consensus 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 cochaperones, the combination of which forms the functional chaperone complex (Arndt *et al.* 2007). In mammals, the *Hsp70*-family cochaperones 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 nonsignificant 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), while thermotolerant *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 (McQuilton *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 cochaperone (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

© 2014 John Wiley & Sons Ltd

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 thermotolerance 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 $F_0$. 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 >4000 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 $F_3$ mass-bred females. Imagoes were collected into mixed-sex cohorts until 24-h prior to the assays where females were aspirated into groups of 20 without $CO_2$. 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.

© 2014 John Wiley & Sons Ltd

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 $F_5$ 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 $CO_2$. 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 $N_2$ 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

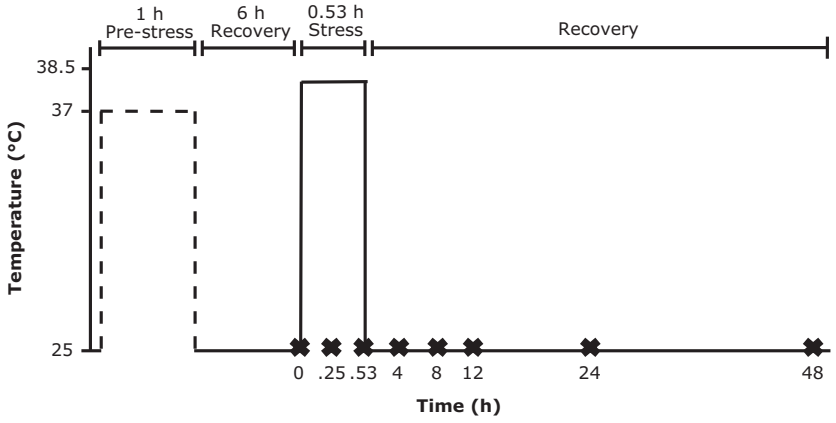


**Fig. 1** Treatment and sampling schematic for the two thermal regimes, nonlethal hardening treatment (dashed box) and subsequent severe thermal stress (solid box). Prior to severe thermal stress, groups of 20 female flies either underwent 1-h exposure to 37 °C followed by 6-h recovery at 25 °C or were 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.

© 2014 John Wiley & Sons Ltd

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) = $2^{(RPL11-\mathrm{TOI})}$. 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

| Gene | Transcript/Subset | Forward primer | Reverse primer |
|---|---|---|---|
| *RPL11* | *RA:RB* | CGATCCCTCCATCGGTATCT | AACCACTTCATGGCATCCTC |
| *stv* | *Pre-RA:RE:RF* | CCCAAAACGCTTACGGATCG | GGGGGCCACTCACCTGAAAA |
| | *Pre-RG:RB:RC* | AAGCGGAAAAGCATTCAAAA | GATGTCGATGTCGGAACCTT |
| | *RA:RE:RF* | CACAGTTCCACACTCCCCAA | GAATCCAAAGGTCGGCTGAA |
| | *RB:RC:RG* | GTCACCAAGCGGAAAAGCAT | CAAAGGTCGGCTTTTGCCTG |
| | *RD* | ACATAGTTGATGTGAAACAGCG | CCAAAGGTCGGCTGTTTTATAATTT |

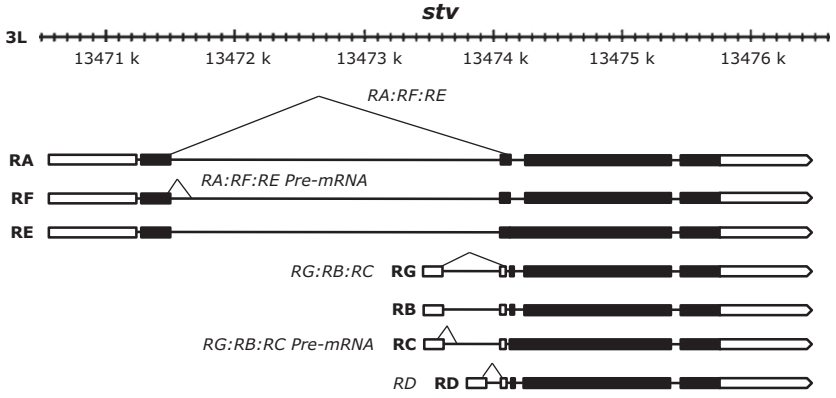


**Fig. 2** *stv* gene model showing the gene region (chromosome 3L:13470641-13476615), long *RA:RE:RF* isoform subset, short isoform subset *RB:RC:RG* and smallest *RD* isoform each derived by alternative start exons. The lines joining exon junctions indicate primer sites for processed (alternatively 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).

© 2014 John Wiley & Sons Ltd

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-*RA: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

| Main effects | d.f. | *F*-value | *P*-value |
|---|---|---|---|
| Treatment | 1 | 417.55 | **<0.0001** |
| Population | 2 | 5.64 | **0.0038** |
| Treatment*population | 2 | 1.70 | 0.1856 |
| Error | 595 | | |

Significant terms are bolded.

© 2014 John Wiley & Sons Ltd

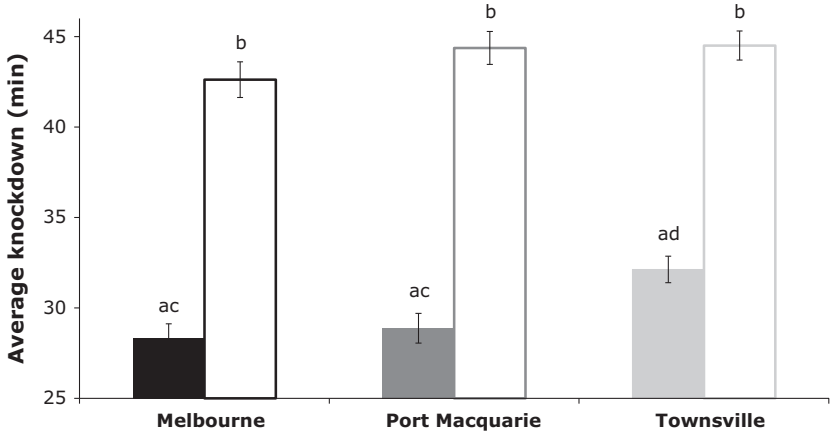


**Fig. 3** Average heat knockdown of individual wild-derived *Drosophila melanogaster* females from three Australian east coast locations representing 'high' latitude (Melbourne, black fill), 'mid' latitude (Port Macquarie grey fill) and 'low' latitude (Townsville light grey fill) for basal tolerance (closed bars) and hardening tolerance (open bars). The hardening treatment on average significantly improved knockdown by around 14 min in all populations (a: basal treatment vs. b: hardening treatment, Two-way ANOVA, $P < 0.0001$), although there were no population differences. Average basal tolerance was higher in the tropical low-latitude population compared to the other populations (c: high and mid latitudes vs. d: low latitude, planned contrasts $P < 0.001$, $P < 0.01$, respectively) while the mid and high latitudes did not significantly differ in average tolerance. Error bars represent ± SE of the mean.

**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

| Main effects | d.f. | *F*-value | *P*-value |
|---|---|---|---|
| *Stv-RF:RA:RE* | | | |
| Treatment | 1 | 2.03 | 1.57 |
| Time | 7 | 11.67 | **<0.0001** |
| Population | 2 | 5.37 | **0.006** |
| Treatment*time | 7 | 1.47 | 0.18 |
| Treatment*pop | 2 | 1.33 | 0.27 |
| Time*pop | 14 | 0.61 | 0.85 |
| *Stv-RG:RB:RC* | | | |
| Treatment | 1 | 2.99 | 0.08 |
| Time | 7 | 31.84 | **<0.0001** |
| Population | 2 | 1.16 | 0.32 |
| Treatment*time | 7 | 32.32 | **<0.0001** |
| Treatment*pop | 2 | 1.42 | 0.25 |
| Time*pop | 14 | 1.12 | 0.35 |
| *Stv-D* | | | |
| Treatment | 1 | 50.47 | **<0.0001** |
| Time | 3 | 30.98 | **<0.0001** |
| Population | 2 | 1.07 | 0.36 |
| Treatment*time | 3 | 27.37 | **<0.0001** |
| Treatment*pop | 2 | 2.98 | 0.06 |
| Time*pop | 6 | 0.97 | 0.45 |

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

© 2014 John Wiley & Sons Ltd

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).

© 2014 John Wiley & Sons Ltd

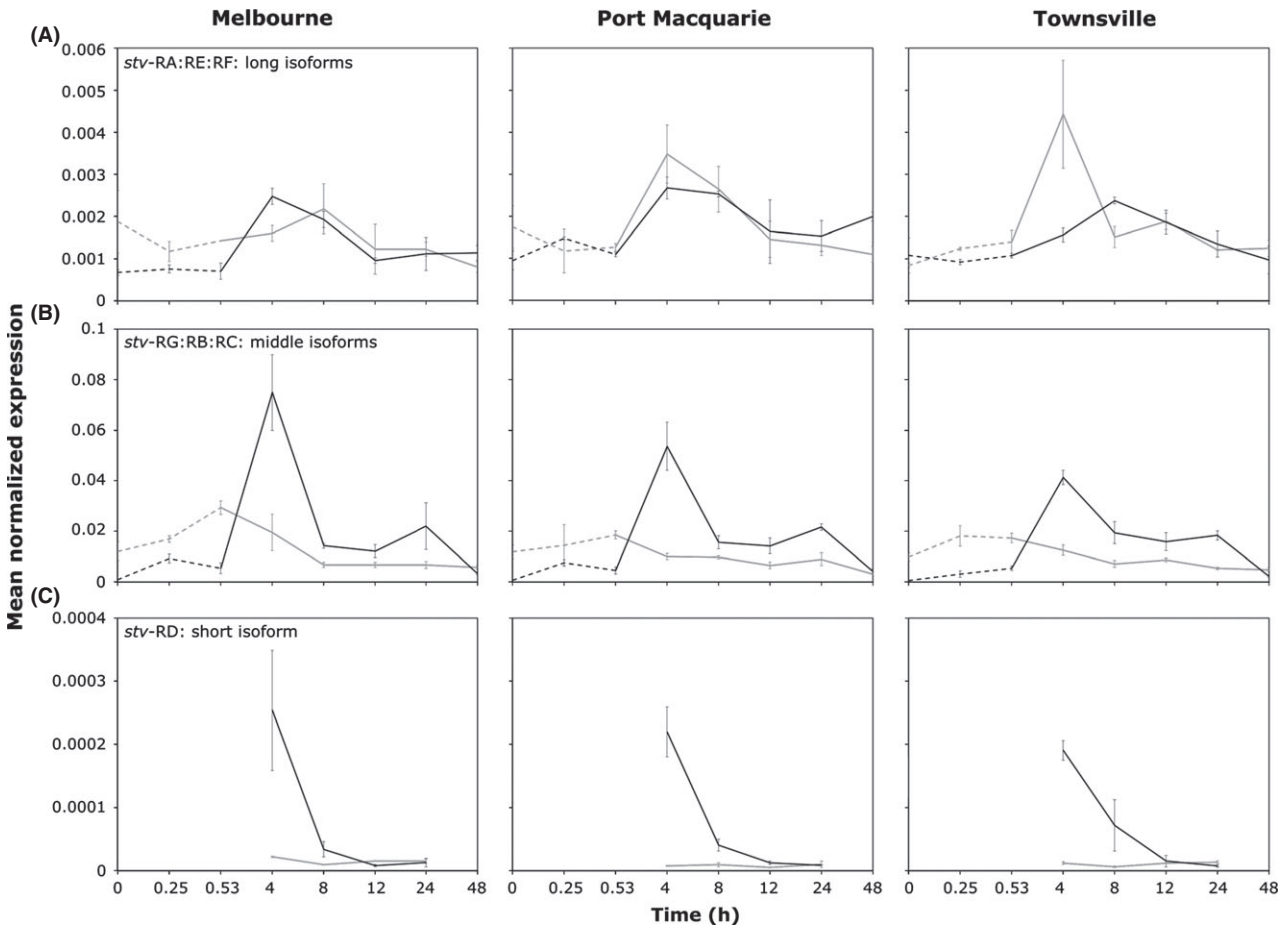


**Fig. 4** Geographic, temporal and isoform variation in expression of *stv* under two different thermal regimes. All isoform/subsets are shown relative *RPL11*, dashed lines = heat shock at 38.5 °C, solid lines = recovery at 25 °C after exposure for 31.5 min. Black lines = unhardened expression, grey 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.

### *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° 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).

© 2014 John Wiley & Sons Ltd

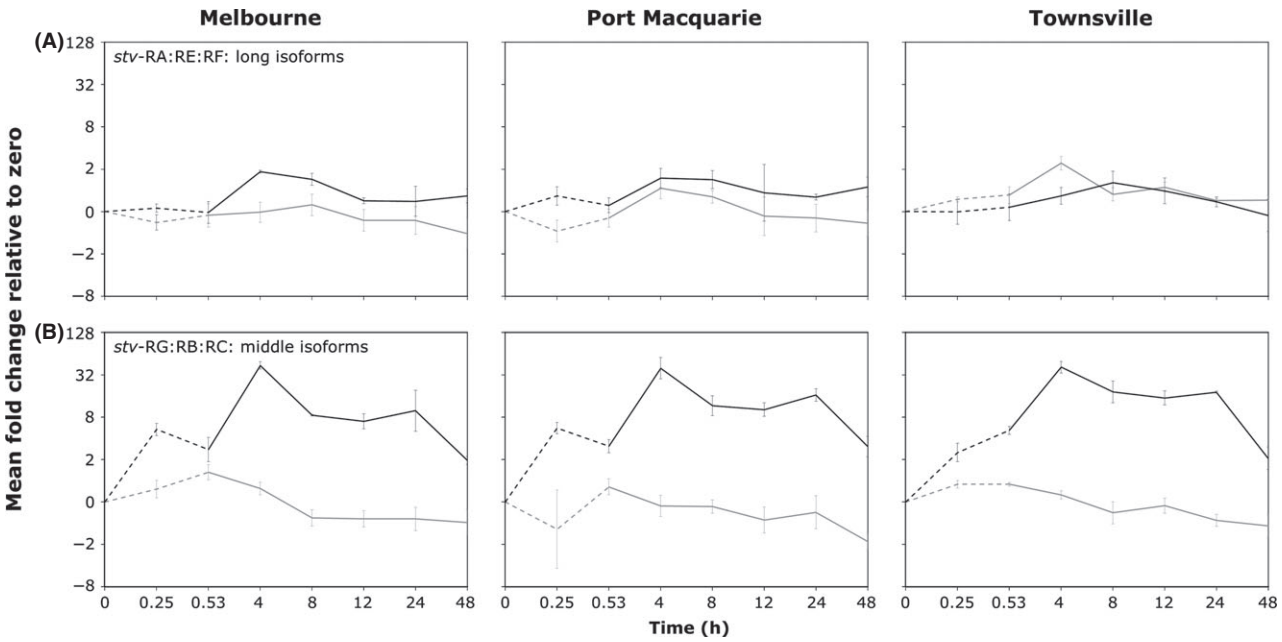


**Fig. 5** *stv* isoform fold change induction relative to prestress. Dashed lines = heat shock at 38.5 °C, solid lines = recovery at 25 °C after exposure for 31.5 min. Black lines = unhardened expression, grey lines = expression following hardening at 37 °C for 1 h prestress. (A) Long isoforms (*RA:RE:RF*). Relative to prestress, the long isoforms were not differentially expressed until recovery. Basal expression peaked at around threefold in the high- and mid-latitude populations (4 and 8 h $P < 0.01$, $P < 0.05$) with no change in hardened flies, whereas in the tropical population, hardening cause the greatest expression at 4-h recovery at around fivefold, while the peak in basal expression was slight at 8-h recovery ($P < 0.0001$ and $<0.05$, respectively). (B) Middle isoforms (*RG:RB:RC*). In the basal treatment, the middle transcripts were rapidly upregulated similarly in all populations from early heat exposure with fold changes consistently between 5- and 10-fold higher than prestress, peaking on average around 80-fold by 4-h recovery, remaining around 30-fold higher at 24-h recovery. The accumulation of transcripts prestress resulting from hardening resulted in slight but attenuated expression, with an average peak about twofold at late stress. Note *RD* is not included as transcripts were not reliably detected until 4-h recovery. 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 an increase 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

© 2014 John Wiley & Sons Ltd

**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*

| Main effects | d.f. | *F*-value | *P*-value |
|---|---|---|---|
| *Stv-RA:RE:RF premRNA* | | | |
| Treatment | 1 | 24.29 | **<0.0001** |
| Time | 7 | 11.70 | **<0.0001** |
| Population | 2 | 0.60 | 0.55 |
| Treatment*time | 7 | 2.11 | **0.04** |
| Treatment*pop | 2 | 3.06 | **0.05** |
| Time*pop | 14 | 0.93 | 0.52 |
| *Stv-RG:RB:RC pre-mRNA* | | | |
| Treatment | 1 | 51.95 | **<0.0001** |
| Time | 7 | 52.82 | **<0.0001** |
| Population | 2 | 1.97 | 0.14 |
| Treatment*time | 7 | 20.43 | **<0.0001** |
| Treatment*pop | 2 | 1.15 | 0.32 |
| Time*pop | 14 | 1.18 | 0.30 |

*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.

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), but decreased to below basal levels almost across the remainder of the time series (Fig. 6B, Table S2, Supporting information).

By contrast in the pre-mRNAs, the basal levels matched the hardened levels around the time that would be expected for the basal group to have become 'hardened' by the first and only heat exposure (i.e. 4-, 8-, 12-h basal vs. hardened FDR corrected $P > 0.05$ all populations, Fig. 6B, Table S2, Supporting information). What is striking, however, is the maintenance of the pre-mRNA pool in hardened flies compared to basal treated flies 48 h after the severe stress and 55 h following the hardening treatment (FDR corrected $P < 0001$ high- and low-latitude populations and $P < 0.05$ mid latitude, Fig. 6B, Table S2, Supporting information). Flies treated with only one stress (basal) showed a considerably steeper decline in pre-*RG:RB:RC* transcripts than those exposed to a double stress (hardening + severe subsequent stress) which resulted in high levels at the end of recovery that were comparable to the mature transcripts (Fig. 6B).

### *Sequence based evidence for the different isoform thermal phenotypes*

To determine whether sequences in the *stv* upstream regulatory region could account for the induction differences observed between the different *stv* isoform subsets, we looked for heat-shock elements (HSEs) known to bind the major transcriptional activator heat-shock factor (HSF) responsible for inducing transcription during heat stress. Manual searches of the genome sequence (*D. melanogaster* genome release version 5.48) immediately upstream of the transcriptional start sites (TSSs) for the three *stv* isoform subsets revealed the presence of three putative HSEs closely matching the canonical binding sequence (nGAAnnTTCnnGAAn) (Gonsalves *et al.* 2011; Table 5). No HSEs were identified upstream of the TSS for the longest isoforms *stv-RA:RE:RF*. 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-

© 2014 John Wiley & Sons Ltd

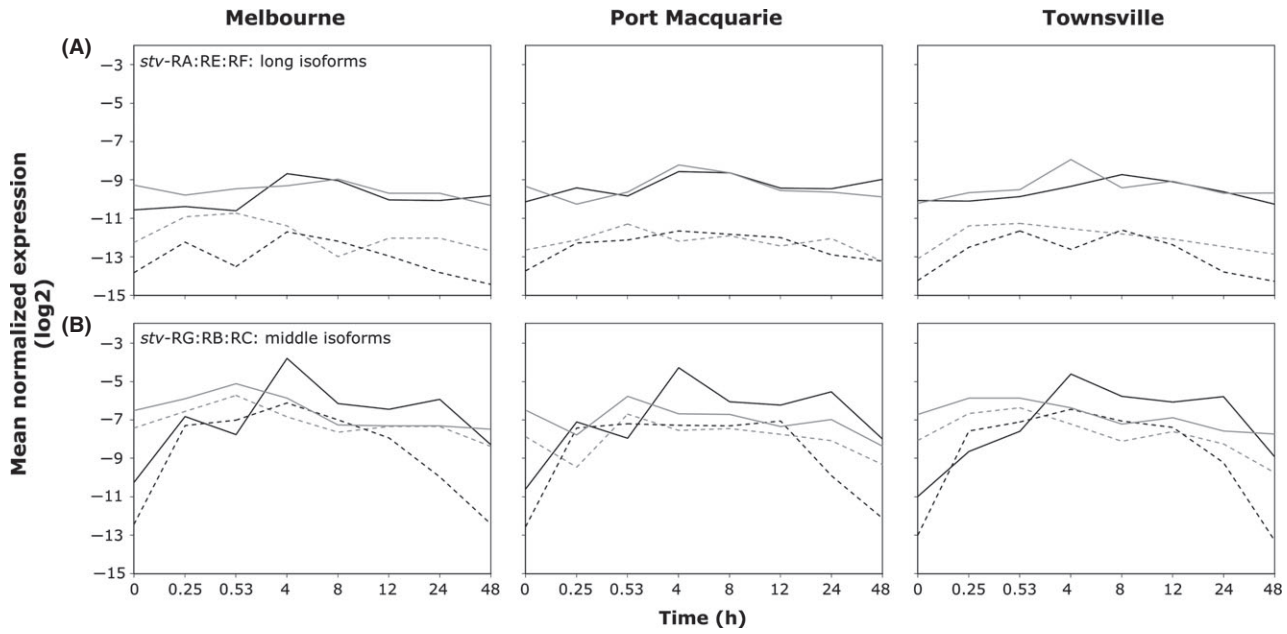


**Fig. 6** *stv* pre- and mature mRNA isoform expression under two thermal regimes. Transcripts are shown log2 transformed relative to *RPL11* to directly contrast primary (dashed lines) and processed (solid lines) mRNA levels. Basal flies are shown in black, hardened are shown in grey. (A) Long isoform 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.

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

| Putative HSE sequence | Chromosomal location | Location relative to *stv* TSS (isoforms) |
|---|---|---|
| GGAACATACGAGAAG | 3L:13470997..13471011 | −2548 to 2534 (RG:RB) |
| TGAAAATTTCTAGAAG | 3L:13472607..13472622 | −938 to 923 (RG:RB) |
| AGAAACTACGAGAAG* | 3L:13473439..13473453 | −106 to 92 (RG:RB) |

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

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.

Previously, we showed that the *stv* transcriptional response to heat stress is complex and identified the thermal induction at the isoform level (Telonis-Scott

© 2014 John Wiley & Sons Ltd

*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 expressed 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 is 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 thermotolerant 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:*

© 2014 John Wiley & Sons Ltd

*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.

## Acknowledgements

We thank Dr Lauren McIntyre for insightful discussion. We are grateful to three anonymous reviewers whose comments improved the manuscript. This work was supported by an

© 2014 John Wiley & Sons Ltd

ARC DECRA Fellowship DE120102575 to M.T.S, and an ARC FT110100951, DP120102045, and Science Industry Endowment Fund (SIEF) grant to C.M.S.

## References


Adelman K, Lis JT (2012) Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. *Nature Reviews Genetics*, **13**, 720–731.

Ali GS, Reddy ASN (2008) Regulation of alternative splicing of pre-mRNAs by stresses. In: *Nuclear Pre-mRNA Processing in Plants* (eds Reddy ASN, Golovkin M), pp. 257–275. Springer-Verlag, Berlin, Germany.

Arndt V, Rogon C, Hohfeld J (2007) To be, or not to be–molecular chaperones in protein degradation. *Cellular and Molecular Life Sciences*, **64**, 2525–2541.

Arndt V, Dick N, Tawo R *et al.* (2010) Chaperone-assisted selective autophagy is essential for muscle maintenance. *Current Biology*, **20**, 143–148.

Arvidsson S, Kwasniewski M, Riano-Pachon DM, Mueller-Roeber B (2008) QuantPrime-a flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinformatics*, **9**, 1471–2105.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate- a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B, Statistical Methodology*, **57**, 289–300.

Bettencourt BR, Hogan CC, Nimali M, Drohan BW (2008) Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. *BMC Biology*, **6**, 1741–7007.

Birch-Machin I, Gao S, Huen D *et al.* (2005) Genomic analysis of heat-shock factor targets in *Drosophila*. *Genome Biology*, **6**, 10.

Blackburn S, van Heerwaarden B, Kellermann V, Sgro CM (2014) Evolutionary capacity of upper thermal limits: beyond single trait assessments. *Journal of Experimental Biology*, **217**, 1918–1924.

Bond U (1988) Heat-shock but not other stress inducers leads to the disruption of a subset of SNRNPS and inhibition of invitro splicing in hela-cells. *Embo Journal*, **7**, 3509–3518.

Bonke M, Turunen M, Sokolova M *et al.* (2013) Transcriptional networks controlling the cell cycle. *G3: Genes, Genomes, Genetics*, **3**, 75–90.

Calabria G, Dolgova O, Rego C *et al.* (2012) Hsp70 protein levels and thermotolerance in *Drosophila subobscura*: a reassessment of the thermal co-adaptation hypothesis. *Journal of Evolutionary Biology*, **25**, 691–700.

Chen YC, Lin SI, Chen YK, Chiang CS, Liaw GJ (2009) The Torso signaling pathway modulates a dual transcriptional switch to regulate tailless expression. *Nucleic Acids Research*, **37**, 1061–1072.

Colinet H, Hoffmann A (2010) Gene and protein expression of *Drosophila Starvin* during cold stress and recovery from chill coma. *Insect Biochemistry and Molecular Biology*, **40**, 425–428.

Colinet H, Siaussat D, Bozzolan F, Bowler K (2013) Rapid decline of cold tolerance at young age is associated with expression of stress genes in *Drosophila melanogaster*. *Journal of Experimental Biology*, **216**, 253–259.

Corell RA, Gross RH (1992) Splicing thermotolerance maintains Pre-mRNA transcripts in the splicing pathway during severe heat shock. *Experimental Cell Research*, **202**, 233–242.

Coulson M, Robert S, Saint R (2005) *Drosophila starvin* encodes a tissue-specific BAG-domain protein required for larval food uptake. *Genetics*, **171**, 1799–1812.

Dahlgaard J, Loeschcke V, Michalak P, Justesen J (1998) Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Functional Ecology*, **12**, 786–793.

Diamond SE, Sorger DM, Hulcr J *et al.* (2012) Who likes it hot? A global analysis of the climatic, ecological, and evolutionary determinants of warming tolerance in ants. *Global Change Biology*, **18**, 448–456.

Doong H, Vrailas A, Kohn EC (2002) What's in the 'BAG'?—a functional domain analysis of the BAG-family proteins. *Cancer Letters*, **188**, 25–32.

Eddison M, Belay AT, Sokolowski MB, Heberlein U (2012) A genetic screen for olfactory habituation mutations in *Drosophila*: analysis of novel foraging alleles and an underlying neural circuit. *PLoS ONE*, **7**, e51684.

Faustino NA, Cooper TA (2003) Pre-mRNA splicing and human disease. *Genes & Development*, **17**, 419–437.

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, **61**, 243–282.

Frankham R (2005) Stress and adaptation in conservation genetics. *Journal of Evolutionary Biology*, **18**, 750–755.

Fujikake N, Nagai Y, Popiel HA *et al.* (2005) Alternative splicing regulates the transcriptional activity of *Drosophila* heat shock transcription factor in response to heat/cold stress. *FEBS Letters*, **579**, 3842–3848.

Gonsalves SE, Moses AM, Razak Z, Robert F, Westwood JT (2011) Whole-genome analysis reveals that active heat shock factor binding sites are mostly associated with non-heat shock genes in *Drosophila melanogaster*. *PLoS ONE*, **6**, e15934.

Graveley BR, Brooks AN, Carlson JW *et al.* (2011) The developmental transcriptome of *Drosophila melanogaster*. *Nature*, **471**, 473–479.

Gubelmann C, Gattiker A, Massouras A *et al.* (2011) GETPrime: a gene- or transcript-specific primer database for quantitative real-time PCR. *Database (Oxford)*, **2011**, bar040.

Guertin MJ, Lis JT (2010) Chromatin landscape dictates HSF binding to target DNA elements. *PLoS Genetics*, **6**, 1001114.

Guertin MJ, Petesch SJ, Zobeck KL, Min IM, Lis JT (2010) *Drosophila* heat shock system as a general model to investigate transcriptional regulation. *Cold Spring Harbor Symposia on Quantitative Biology*, **75**, 1–9.

van Heerwaarden B, Sgro CM (2013) Multivariate analysis of adaptive capacity for upper thermal limits in *Drosophila simulans*. *Journal of Evolutionary Biology*, **26**, 800–809.

Hoffmann AA, Sgro CM (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–485.

Hoffmann AA, Weeks AR (2007) Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica*, **129**, 133–147.

Hoffmann AA, Anderson A, Hallas R (2002) Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters*, **5**, 614–618.


© 2014 John Wiley & Sons Ltd

IPCC (2013) Summary for policymakers. In Climate Change 2013: The Physical Science Basis Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.

Jensen LT, Nielsen MM, Loeschcke V (2008) New candidate genes for heat resistance in *Drosophila melanogaster* are regulated by HSF. *Cell Stress and Chaperones*, **13**, 177–182.

Jensen LT, Cockerell FE, Kristensen TN *et al.* (2010) Adult heat tolerance variation in *Drosophila melanogaster* is not related to Hsp70 expression. *Journal of Experimental Zoology. Part A: Ecological Genetics and Physiology*, **313**, 35–44.

Jolly C, Vourc'h C, Robert-Nicoud M, Morimoto RI (1999) Intron-independent association of splicing factors with active genes. *Journal of Cell Biology*, **145**, 1133–1143.

Kellermann V, Overgaard J, Hoffmann AA *et al.* (2012) Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences*, **109**, 16228–16233.

Keren H, Lev-Maor G, Ast G (2010) Alternative splicing and evolution: diversification, exon definition and function. *Nature Reviews Genetics*, **11**, 345–355.

Kim YE, Hipp MS, Bracher A, Hayer-Hartl M, Hartl FU (2013) Molecular chaperone functions in protein folding and proteostasis. *Annual Review of Biochemistry*, **82**, 323–355.

Krebs RA, Feder ME (1998) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *Journal of Insect Physiology*, **44**, 1091–1101.

Krebs RA, Feder ME, Lee J (1998) Heritability of expression of the 70 KD heat-shock protein in *Drosophila melanogaster* and its relevance to the evolution of thermotolerance. *Evolution*, **52**, 841–847.

Kristensen TN, Loeschcke V, Hoffmann AA (2007) Can artificially selected phenotypes influence a component of field fitness? Thermal selection and fly performance under thermal extremes. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 771–778.

Lindquist S (1980) Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. *Developmental Biology*, **77**, 463–479.

Lindquist S, Craig EA (1988) The heat-shock proteins. *Annual Review of Genetics*, **22**, 631–677.

Marden JH (2008) Quantitative and evolutionary biology of alternative splicing: how changing the mix of alternative transcripts affects phenotypic plasticity and reaction norms. *Heredity*, **100**, 111–120.

Mastrangelo AM, Marone D, Laido G, De Leonardis AM, De Vita P (2012) Alternative splicing: enhancing ability to cope with stress via transcriptome plasticity. *Plant Science*, **185**, 40–49.

McQuilton P, St Pierre SE, Thurmond J (2012) FlyBase 101–the basics of navigating FlyBase. *Nucleic Acids Research*, **40**, 29.

Moribe Y, Niimi T, Yamashita O, Yaginuma T (2001) Samui, a novel cold-inducible gene, encoding a protein with a BAG domain similar to silencer of death domains (SODD/BAG-4), isolated from Bombyx diapause eggs. *European Journal of Biochemistry*, **268**, 3432–3442.

Nilsen TW, Graveley BR (2010) Expansion of the eukaryotic proteome by alternative splicing. *Nature*, **463**, 457–463.

Pagliuca MG, Lerose R, Cigliano S, Leone A (2003) Regulation by heavy metals and temperature of the human BAG-3 gene, a modulator of Hsp70 activity. *FEBS Letters*, **541**, 11–15.

Pal S, Gupta R, Davuluri RV (2012) Alternative transcription and alternative splicing in cancer. *Pharmacology & Therapeutics*, **136**, 283–294.

Perisic O, Xiao H, Lis JT (1989) Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. *Cell*, **59**, 797–806.

Rosati A, Ammirante M, Gentilella A *et al.* (2007) Apoptosis inhibition in cancer cells: a novel molecular pathway that involves BAG3 protein. *The International Journal of Biochemistry & Cell Biology*, **39**, 1337–1342.

Sgro CM, Overgaard J, Kristensen TN *et al.* (2010) A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from eastern Australia. *Journal of Evolutionary Biology*, **23**, 2484–2493.

Sorensen JG, Michalak P, Justesen J, Loeschcke V (1999) Expression of the heat-shock protein HSP70 in *Drosophila buzzatii* lines selected for thermal resistance. *Hereditas*, **131**, 155–164.

Sorensen JG, Nielsen MM, Kruhoffer M, Justesen J, Loeschcke V (2005) Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress and Chaperones*, **10**, 312–328.

Telonis-Scott M, Hoffmann AA, Sgro CM (2011) The molecular genetics of clinal variation: a case study of ebony and thoracic trident pigmentation in *Drosophila melanogaster* from eastern Australia. *Molecular Ecology*, **20**, 2100–2110.

Telonis-Scott M, van Heerwaarden B, Johnson TK, Hoffmann AA, Sgro CM (2013) New levels of transcriptome complexity at upper thermal limits in wild *Drosophila* revealed by exon expression analysis. *Genetics*, **195**, 809–830.

Teves SS, Henikoff S (2011) Heat shock reduces stalled RNA polymerase II and nucleosome turnover genome-wide. *Genes & Development*, **25**, 2387–2397.

Vazquez J, Pauli D, Tissières A (1993) Transcriptional regulation in *Drosophila* during heat shock: a nuclear run-on analysis. *Chromosoma*, **102**, 233–248.

Vermeulen CJ, Sørensen P, Kirilova Gagalova K, Loeschcke V (2013) Transcriptomic analysis of inbreeding depression in cold-sensitive *Drosophila melanogaster* shows upregulation of the immune response. *Journal of Evolutionary Biology*, **26**, 1890–1902.

Werren JH (1997) Biology of wolbachia. *Annual Review of Entomology*, **42**, 587–609.

Westwood JT, Clos J, Wu C (1991) Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. *Nature*, **353**, 822–827.

Yost HJ, Lindquist S (1986) RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell*, **45**, 185–193.

Yost HJ, Petersen RB, Lindquist S (1990) RNA metabolism-strategies for regulation in the heat-shock response. *Trends in Genetics*, **6**, 223–227.

---

M.T.S. and C.M.S. designed the experiments, A.S.C. performed the research, M.T.S. analysed the phenotype and transcript data and wrote the manuscript, T.K.J.

© 2014 John Wiley & Sons Ltd

performed the HSE analyses, and C.M.S., A.S.C. and T.K.J. contributed to the manuscript.

## 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.

© 2014 John Wiley & Sons Ltd