MONASH University

The transcriptional regulation of plastic responses to stress in *Drosophila melanogaster*

Allannah S. Clemson
BSc(Hons), DipLang

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Abstract

Climate change is enhancing the fluctuations in weather conditions including increasing temperature and precipitation variability. This imposes a great deal of stress on terrestrial arthropods such as *Drosophila*, which rely on the environment to maintain homeostasis. To counteract these deleterious effects, phenotypic plasticity can enable species to maintain their optimal fitness and allow them to persist in an otherwise harmful environment. While some work has focused on understanding the extent to which species can use phenotypic plasticity to mediate climatic change, little progress has been made on elucidating the molecular mechanisms facilitating this adaptive strategy. Therefore, this thesis aims to address this deficit and attempts to link plastic responses at the transcript level to plastic responses at the quantitative trait level.

My goal was first to understand the extent to which two populations of *D. melanogaster* from ends of the east Australian latitudinal cline could elicit plastic responses when exposed to different developmental temperatures and humidity conditions. In total, I measured six quantitative traits; fecundity, body size, viability, heat and cold tolerance were examined on flies developed at six different temperatures (18^{o}C – 30^{o}C), and desiccation resistance on flies exposed to different stress pre-treatments. All six quantitative traits were plastic, and all, except viability, differed between the two populations. However, only two (fecundity and desiccation resistance) showed evidence for geographic variation in plasticity.

I then examined a subset of candidate genes for thermal tolerance and desiccation resistance to characterise their expression profiles and determine the extent to which they mirrored the phenotypic results. Despite the expression patterns of many of the 23 thermal candidate genes and one of the 12 desiccation resistance candidate genes differing between the populations, I did not find evidence for genetic variation maintaining expression plasticity. However, given the complex physiological architecture of desiccation resistance and, to a lesser extent, heat tolerance, my results provide the first insights into the molecular basis of desiccation plasticity, and make a significant contribution to understanding the molecular mechanisms underpinning environmental adaptation.

General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in a peer reviewed journal and 1 publication to be submitted. The core theme of the thesis is the *Evolution of Environmental Adaptation*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the *School of Biological Sciences* under the supervision of *Dr. Marina Telonis-Scott* and *Dr. Carla Sgró*.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

Thesis Chapter	Publication Title	Publication Status	Contribution
2	Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts	Accepted: *Journal of Evolutionary Biology*	70%
3	Hardening but not population divergence impacts temporal expression profiles of candidate genes for desiccation resistance in *Drosophila melanogaster*	For Submission	70%

Other work produced during my thesis period:

Telonis-Scott, M., **Clemson, A. S**., Johnson, T. K., Sgró, C. M. (2014) Spatial analysis of gene regulation reveals new insights into the molecular basis of upper thermal limits. Molecular Ecology, 23(24), 6135-6151.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  **Date:** 19/12/2016

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:  **Date:** 19/12/2016

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Thank-you to my supportive parents and brother for your love and encouragement. You are the reason I keep furthering my education and will likely continue to in the future. To my closest and dearest friends Thalia Prum, Shannon Everett and Alisha McLennan for the friendship and needed distraction from writing. I would not be the person I am today without you three. Last, but not least, my biggest thanks go to my incredible partner Tom McGarr. You've always been there for me, turned my frown upside down, kept me smiling and often been my companion on late nights in the lab. You truly are my rock and I appreciate and love you.

CHAPTER 1

Introduction

1.1 Overcoming environmental change

Global anthropogenic climate change is now occurring at an unprecedented pace (IPCC 2014) resulting in increased fluctuations in temperature and rainfall (Fung *et al.* 2011; Sanderson *et al.* 2011). Both environmental temperature and water availability impact the abundance and distribution of species, potentially leaving them vulnerable to the enhanced fluctuations that climate change brings (Chown *et al.* 2011; Cossins & Bowler 1987). Understanding a species' capacity to withstand environmental change is therefore crucial in assessing the impact climate change will have on biodiversity.

When organisms are unable to mitigate harmful environmental conditions via migration or dispersal, adaptation can allow them to persist in their original environmental range (Hoffmann 2010; Hoffmann & Sgrò 2011; Williams *et al.* 2008). Climatic adaptation can occur either by evolutionary or plastic responses (Williams *et al.* 2008). Adaptive evolutionary responses to environmental change are cross-generational and result in frequency shifts towards advantageous alleles over time (Lynch & Walsh 1998). While there is increasing evidence for rapid species evolution in the face of climate change, within a time scale of decades (Nussey *et al.* 2005; Reale *et al.* 2003), and even years (Bradshaw & Holzapfel 2001), this process may still be too slow to allow species to respond adaptively in the face of rapid climate change (Bradshaw & Holzapfel 2006).

Unlike evolution, phenotypic plasticity can be rapid, occurring within the lifetime of the organism (Nicotra *et al.* 2010; Sgrò *et al.* 2016). Plasticity allows an individual organism to produce multiple phenotypes in response to environmental cues, thus reducing the need for evolutionary shifts (West-Eberhard 2003). Because of this, it may act as a buffer in a rapidly changing environment to potentially counteract any deleterious effects these changes might have on organismal performance. For example, in Great Tits (*Parus major*), plasticity in reproductive timing allows individuals to match their breeding times with the life-cycle of their prey (Nussey *et al.* 2005). Over time, warmer springs have induced a shift in the growth of their caterpillar prey towards earlier maturation. Phenotypic plasticity in breeding time acts to alleviate any mismatch that this seasonality might cause (Nussey *et al.* 2005). In enabling the organism to persist, plasticity as a trait can be adaptive (Ghalambor *et al.* 2007; Gotthard & Nylin 1995; Hoffmann 2010; West-Eberhard 1989). In the face of

environmental change, it might prolong the species' survival, by allowing sufficient time for an adaptive genetic response to evolve.

Until recently, climate adaptation research has focussed largely on physiological thermal limits (i.e. upper and lower limits) and how these might impact species persistence over time (Hoffmann 2010). However, global temperature is not the only ecological process affected by climate change. Receiving somewhat less attention are impacts on global precipitation levels which are set to increase in the tropic and decrease in the sub-tropical and temperate regions under projected climate change, albeit less predictably than temperature projections (Sanderson *et al.* 2011). Importantly, recent studies have suggested that the combined effects of both precipitation and temperature can also have significant effects on environmental adaptation (Bubliy *et al.* 2013; Kellermann *et al.* 2012; Kellermann *et al.* 2009; Sanderson *et al.* 2011), there is a pressing need to examine the effect of multiple climatic stresses.

1.2 Climatic gradient (clinal) studies: Understanding adaptation to environmental change

One commonly proposed method by which adaptation across heterogeneous environments can occur is spatially varying selection, where intraspecific genetic variation can be maintained despite the homogenising effect of gene flow (Endler 1977; Felsenstein 1976). When environmental heterogeneity occurs across a wide enough geographical range, intraspecific variation for traits can form clines (Endler 1977; Huxley 1938; Slatkin 1973). While this variation can sometimes be the result of underlying population structure, spatially varying selection driven by environmental factors (such as temperature) is generally thought to be the main driver (David *et al.* 1977; Dobzhansky 1970; Endler 1977; Stalker & Carson 1947). Clinal variation can therefore be used to infer spatially varying selection on traits linked to climatic adaptation (Endler 1977; Hoffmann & Weeks 2007; Sgrò *et al.* 2010; Telonis-Scott *et al.* 2014). When populations along a latitudinal gradient are tested under common conditions and the differences in trait of interest persist, this provides a link to genetic evolutionary responses to environmental conditions (Hoffmann & Weeks 2007; Somero 2010). Thus, studying clinal patterns allows a species' adaptive responses to

climate selection to be understood, and provides insight into their susceptibility to climate change (Endler 1977).

The model species *Drosophila melanogaster* has frequently been used to examine the role of spatially varying selection in climatic adaptation, as its broad geographic range means that populations are exposed to quite different environmental conditions. As such, latitudinal clines for many quantitative traits including morphology (Azevedo *et al.* 1998; Telonis-Scott *et al.* 2011), stress resistance (Hoffmann *et al.* 2002; van Heerwaarden *et al.* 2012), protein synthesis rates (Cockerell *et al.* 2014), chromosome inversions (Stalker 1976; Umina *et al.* 2005) and gene expression (Cockerell *et al.* 2014; Swindell *et al.* 2007; Whitehead & Crawford 2006) have previously been described. Such studies provide indirect evidence for genetic adaptation in response to climatic selection and highlight both the physiological and ecological factors contributing to the evolution of environmental adaptation (Sgrò *et al.* 2010; van Heerwaarden *et al.* 2012).

Many studies characterising clinal patterns involve so-called common garden experiments that enable the relative importance of evolved genetic components of a trait to be examined. When these studies also involve multiple experimental environments (e.g. different rearing temperatures), the relative importance of plasticity and evolution in geographic adaptation can also be assessed (Hoffmann *et al.* 2005). Evolution and plasticity are not mutually exclusive and as such, genetic variation for plasticity – formally referred to as genotype-by-environment interactions (GEI) – is widespread (DeWitt & Scheiner 2004; Fallis *et al.* 2014; Gutteling *et al.* 2007; Winterhalter & Mousseau 2007). Significant GEI indicates that the magnitude of a population's plastic response can be under selection and is environment specific (Price *et al.* 2003; Scheiner 1993).

1.3 Phenotypic plasticity: minimising the negative effects of environmental change

Plasticity is a ubiquitous process (Pigliucci, 2001) and is conserved among species with both restricted and widespread distributions (Overgaard et al., 2011).

Furthermore, it is sometimes seen as playing a more important role than evolution in responses to rapid environmental change (Hoffmann & Sgró, 2011). For example, a decrease in body size in response to changing winter conditions was found in a population of Soay sheep (*Ovis aries*) inhabiting the island of Hirta. This decrease was attributed to plasticity and occurred despite the fitness advantage heavier individuals have over lighter ones (Ozgul et al., 2009). Plasticity has also been found to be more important in changes in bird breeding dates (Gienapp et al. , 2008) and increased body size in yellow-bellied marmots (Ozgul et al. , 2010) in response to climate change.

Phenotypic plasticity for a trait can be measured in one of two ways: either by short-term or long-term exposure to sub-lethal stress followed by lethal assays (Hoffmann *et al.* 2003a). Short-term assays involve exposure to sub lethal stress for minutes or hours and are referred to as hardening (Cossins & Bowler 1987). Conversely, long-term assays expose the organism for days or weeks to moderate stress within their normal range and are commonly termed acclimation (Hoffmann *et al.* 2003a). Typically, individuals exposed to either of these treatments have increased stress tolerance compared to their non-exposed (basal) counterparts (reviewed in Hoffmann et al. 2003). Comparing hardened (plastic) and basal (genetic) treatments can provide information about the relative capacity of populations or species to exhibit a plastic response to environmental stress (Kellett *et al.* 2005).

It is generally thought that populations living in more variable environments, such as temperate populations, show greater levels of phenotypic plasticity than those living in more stable conditions such as the tropics (Angilletta, 2009). However, Sgró *et al.* (2010) found that tropical *D. melanogaster* populations had larger plastic responses for thermotolerance than their temperate counterparts, despite living in a less variable environment. They suggest that rather than operating on the organism as a whole, phenotypic plasticity might instead be trait specific and less related to habitat heterogeneity. This is also supported by work on *D. serrata* where tropical populations have higher plasticity in developmental rate but lower plasticity in body size compared to temperate populations (Liefting et al., 2009). Thus, while increasing evidence suggests that plasticity plays an important role in climatic adaptation, we have very little insight into how phenotypic plasticity is mediated at the cellular level (Telonis-Scott *et al.* 2013).

1.4 Molecular mechanisms underpinning environmental adaptation

Previously, climatic adaptation studies concerned with phenotypic plasticity have focused on assessing quantitative traits – traits considered important to environmental adaptation such as heat, cold and desiccation resistance (Colinet & Hoffmann 2012; Hoffmann et al. 2005; Sgró et al. 2010), or those that form clines across latitudinal gradients such as fecundity (Klepsatel et al. 2013) and body size (David et al. 1997). However, increasingly there has been a shift towards understanding plasticity of the transcriptome; that is, characterising the patterns of gene expression that are observed between environmental conditions and experimental populations (Levine et al. 2011; Zhao et al. 2015).

Whole-transcriptome approaches frequently uncover hundreds or thousands of genes implicated in environmental adaptation. For example, Hodgin-Davis et al. (2012) characterised the transcriptome plasticity differences in five strains of *Saccharomyces cerevisiae* exposed to different copper concentrations. They identified 1,606 genes with significant GEI. Furthermore, Grishkevich et al. (2012) found 198 genes with significant GEI between five *Caenorhabditis elegans* strains exposed to different environmental conditions. Studies attempting to examine this in *D. melanogaster* have also identified suites of genes that could potentially play a role in climatic adaptation. Using two outbred populations from the Australian east-coast cline developed at two temperatures, Levine et al. (2011) identified 56 genes that showed evidence of GEI. In a similar study on North American clinal populations, again developed at two temperatures, Zhao et al. (2015) identified 264 significant GEI genes. Furthermore, many of these GEI genes correspond to those known to differ in transcript abundance between African and European populations (Hutter et al. 2008), and implicated in thermal plasticity (Chen et al. 2015). Not only are these candidates for thermal plasticity variation but they also appear to be important in environmental adaptation.

Integrating transcriptomic responses with a species' phenotypic stress response is only in its infancy (Telonis-Scott et al., 2013). This means that until recently, studies attempting to link the molecular mechanisms underpinning phenotypic plasticity in stress traits have generally focused on a few, stress related candidate genes (Bettencourt et al., 2002, Frydenberg et al., 2003, McColl and McKechnie, 1999) and often neglect to characterise the phenotypic response at all. Furthermore, it has

resulted in an overwhelming bias towards heat tolerance traits, likely driven by early work in describing the heat-shock response (Lindquist, 1986). We still know very little about the cellular response to desiccation stress (Telonis-Scott *et al.* 2016) and nothing at all about the molecular responses to desiccation plasticity.

1.5 Aims and thesis structure

This thesis aimed to link plastic responses at the transcript level to plastic responses at the quantitative level. It is comprised of two empirical chapters, followed by a final discussion and future directions chapter. The empirical chapters are written in the style of journal article with tables and figures embedded into the text. The results chapters are as follows:

Chapter 2 describes the use of two climatically divergent *D. melanogaster* populations developed at six different rearing temperatures to examine reaction norm variation at the transcript and quantitative trait level. A comprehensive suite of traits was examined including five quantitative traits (3 morphological and 2 fitness) and 23 transcript-level traits. This chapter uses developmental acclimation (long-term pre-exposure) to elicit plastic responses. This work has been accepted for publication in the *Journal of Evolutionary Biology*.

Chapter 3 uses the same *D. melanogaster* populations used in chapter 2 and characterises each population's innate (basal) and plastic response to desiccation stress at a phenotypic level, and at a molecular level. This chapter uses a candidate gene approach (12 candidate desiccation resistance genes) to try to understand the molecular mechanisms driving the differences in desiccation plasticity observed between the two populations. For this chapter, the plastic response was elicited using hardening (shot-term pre-exposure) treatment.

The final chapter (Chapter 4) brings together the main findings from the two empirical chapters and provides a general discussion, followed by suggested future research directions.

Declaration for Thesis Chapter 2

"Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts"

This thesis chapter is in publication format for the *Journal of Evolutionary Biology*

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Contribution (%)
Experimental design, execution of experimental work, data analysis, manuscript writing	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Carla M. Sgro	Experimental design, manuscript editing	15%
Marina Telonis-Scott	Experimental design, manuscript writing	15%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature  **Date** 19/12/2016

Main Supervisor's Signature  **Date** 19/12/2016

CHAPTER 2

Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts

2.1 Abstract

The flexibility afforded to genotypes in different environments by phenotypic plasticity is of interest to biologists studying thermal adaptation because of the thermal lability of many traits. Differences in thermal performance and reaction norms can provide insight into the evolution of thermal adaptation to explore broader questions such as species distributions and persistence under climate change. One approach is to study the effects of temperature on fitness, morphological and more recently gene expression traits in populations from different climatic origins. The diverse climatic conditions experienced by *D. melanogaster* along the eastern Australian temperate-tropical gradient is ideal given the high degree of continuous trait differentiation, but reaction norm variation has not been well studied in this system. Here, we reared a tropical and temperate population from the ends of the gradient over six developmental temperatures and examined reaction norm variation for five quantitative traits including thermal performance for fecundity, and reaction norms for thermotolerance, body size, viability and 23 transcript-level traits. Despite genetic variation for some quantitative traits, we found no differentiation between the populations for fecundity thermal optima and breadth, and the reaction norms for the other traits were largely parallel, supporting previous work suggesting that thermal evolution occurs by changes in trait means rather than by reaction norm shifts. We examined reaction norm variation in our expanded thermal regime for a gene set shown to previously exhibit GxE for expression plasticity in east Australian flies, as well as key heat shock genes. While there were differences in curvature between the populations suggesting a higher degree of thermal plasticity in expression patterns than for the quantitative traits, we found little evidence to support a role for genetic variation in maintaining expression plasticity.

2.2 Introduction

Phenotypic plasticity is the ability of a single genotype to generate diverse phenotypes in response to environmental variation (West-Eberhard 2003). This flexibility is widespread and is predicted to be adaptive when the altered phenotype parallels the native optimum phenotype, but how plasticity impacts adaptive evolution is debatable because of limited empirical data (Ghalambor *et al.* 2007). Temperature is a ubiquitous factor affecting organismal fitness and distributions, which are often limited to specific thermal ranges to maintain biochemical stability and metabolic activity (Cossins & Bowler 1987; David & Tsacas 1981; Hochachka & Somero 2002). This is particularly true for ectotherms, as their thermal environment dictates the maintenance of homeostasis, body temperature, adult size and ultimately fitness (Angilletta Jr 2009; Angilletta Jr & Dunham 2003; Huey 1982; Stevenson 1985). The impact of enzyme thermodynamics on thermal sensitivity underpins opposing hypotheses on the evolution of optimal phenotypes in warm and cold adapted organisms where it is proposed that 'hotter is better', because higher temperatures expedite chemical reactions (Angilletta Jr *et al.* 2010). The interplay between temperature and plasticity is highly topical in thermal biology research, with a focus on understanding the mechanisms of plasticity and role in phenotypic evolution, population/species diversity and distributions, and persistence in a changing climate (reviewed in Sgrò *et al.* 2016).

Thermal reaction norms are typically used to describe and compare the effects of temperature (Huey & Stevenson 1979; Scheiner 1993), and are a useful measure of the scale and direction of a plastic response. While reaction norms describe the effect of temperature on final trait values, a second measure of thermal plasticity, performance during thermal exposure, can be illustrated by the thermal performance curve (Angilletta Jr 2009; Kingsolver *et al.* 2004). The properties defined by the performance curve permit biologically important inferences regarding the thermal optimum (T_{opt}), the temperature at which performance is maximal (P_{max}), and performance breadth and thermal limits (Angilletta Jr *et al.* 2002). These graphical and mathematical models can be applied empirically to study the evolution of thermal plasticity using different approaches (summarized in Fragata *et al.* 2016). A common approach in ectotherms is to compare developmental acclimation across a thermal range in populations/species from different climatic origins (Berger *et al.* 2013; Fallis

et al. 2014; Klepsatel *et al.* 2013; Liefting *et al.* 2009; Phillips *et al.* 2014; Trotta *et al.* 2006; Yamahira *et al.* 2007; Zhao *et al.* 2015). In this framework differences in the slopes and/or thermal breadth and optima of reaction norms may provide evidence for geographic (i.e. genetic) variation in the direction and/or the magnitude of plasticity (Kingsolver *et al.* 2004). Similarly, non-additive effects of the genotype in different environments known as genotype-by-environment interactions (GxE) can indicate genetic variation for plasticity (DeWitt & Scheiner 2004; Price *et al.* 2003; Scheiner 1993).

Drosophila melanogaster is ideal for studying thermal adaptation; this species is viable across a wide temperature range (reviewed in Hoffmann 2010), and exhibits parallel clines in quantitative fitness and morphological traits, chromosome inversions, DNA polymorphisms, gene expression, and other traits (Adrion *et al.* ; Azevedo *et al.* 1998; David *et al.* 1977; Gibert & Huey 2001; Hoffmann & Weeks 2007; James *et al.* 1997; Land *et al.* 1999). Clinal patterns may arise where there are spatially continuous changes in traits, and taking population structure into account, can reflect natural selection to climatic conditions such as temperature (Endler 1977; Hoffmann & Weeks 2007). The eastern Australian temperate-tropical latitudinal gradient is an excellent resource to study intraspecific local adaptation given the diverse local climates (Hoffmann & Weeks 2007), clines in thermal tolerance (Hoffmann *et al.* 2002; Sgrò *et al.* 2010) including a thermal candidate gene *Hsrω* (Cockerell *et al.* 2014), gene expression (Lee *et al.* 2011; Telonis-Scott *et al.* 2011) and thermal phenotypic plasticity (Sgrò *et al.* 2010; Telonis-Scott *et al.* 2011). Rapid latitudinal shifts in DNA polymorphisms on the Australian east coast may also serve as indicators for climate change (Umina *et al.* 2005).

Drosophila thermal plasticity research more generally has focused on quantitative phenotypes including morphometrical traits such as bristle number, body size, body colouration and ovariole number (Delpuech *et al.* 1995; Gibert *et al.* 2004; Klepsatel *et al.* 2013; Moreteau *et al.* 2003; Morin *et al.* 1999), fitness traits thermal performance for fecundity (Klepsatel *et al.* 2013) and impacts of thermal regime on thermotolerance (Hoffmann & Watson 1993; Overgaard *et al.* 2011). Recent high throughput –omics platforms assess tens of thousands of transcript-level phenotypes simultaneously, and there has been increasing interest in genome-wide thermal expression plasticity

correlated with geographic origin, and in the role of spatially varying selection in maintaining transcriptome-level variation between populations and species (Levine *et al.* 2011; Zhao *et al.* 2015). Genome-wide reaction norms have also been used to identify genes with common regulatory architecture and functional roles (Chen *et al.* 2015).

Thermal variation is also thought to impact performance; Levins (1968), proposed that widespread species experience greater thermal heterogeneity than restricted species, leading to predictions of broader performance breadth in temperate verses tropical *Drosophila* (Overgaard *et al.* 2011). The limited empirical data however is inconclusive; several studies show that while *Drosophila* quantitative traits are highly plastic, differences in some fitness traits are driven by trait mean values rather than differences in reaction norms (plasticity) (Cooper *et al.* 2012; Delpuech *et al.* 1995; Hoffmann & Watson 1993; Klepsatel *et al.* 2013; Overgaard *et al.* 2011). Conversely for morphology, plasticity may be a factor underpinning differences between tropical and temperate *Drosophila* in traits such as size colouration (David *et al.* 1997; Morin *et al.* 1999). Molecular phenotypes are also highly plastic; developmental acclimation impacted over 80% of the expressed genes over a broad thermal range in inbred *D. melanogaster* adults (Chen *et al.* 2015). However comparative thermal plasticity expression data in outbred populations from different climatic origins is so far limited to two extreme rearing temperatures (Levine *et al.* 2011; Zhao *et al.* 2015). There is evidence however for GxE for a number of genes suggesting the maintenance of genetic variation for thermal plasticity related to latitude in *D. melanogaster* (Levine *et al.* 2011; Zhao *et al.* 2015), but to a lesser extent in *D. simulans* (Zhao *et al.* 2015). However the limited number of thermal environments used in these studies provides limited insight into the relative contribution of plasticity versus trait mean divergence in climatic adaptation (Sgrò *et al.* 2016).

In the current study we utilize the well-established 'cline-end' sampling strategy (e.g. Hoffmann & Watson 1993; Levine *et al.* 2011; Morin *et al.* 1999; Trotta *et al.* 2006) to comprehensively survey thermal plasticity across a wide range of temperatures in a tropical and temperate population of *D. melanogaster* from eastern Australia. Reaction norm variation in quantitative and molecular traits across several thermal environments has not been well studied and we address this by assessing a test set

of 28 fitness, morphological and molecular traits in outbred populations from opposing ends of the same climatic gradient and ask if mean performance and reaction norms differ according to climatic origin. For the fitness trait fecundity, we examined key parameters of thermal performance, thermal optima, maximum output and breadth. We examined stress resistance variation using standard measures of heat and cold tolerance, and examined egg-to-adult viability and body size reaction norms. Utilising our wider thermal range, we also examined a test set of genes identified from whole transcriptome studies that have previously shown evidence for geographic and/or GxE for thermal plasticity (Chen *et al.* 2015; Levine *et al.* 2011; Zhao *et al.* 2015) to explore potential patterns of spatial selection maintaining genetic variation for molecular plasticity in a comparative framework.

2.3 Materials and methods

2.3.1 *D. melanogaster* collection and maintenance

D. melanogaster were collected using banana baits from Melbourne (temperate; 37.8136° S, 144.9631° E) and Innisfail (tropical; 17.5236° S, 146.0292° E), Australia in March and May 2013 respectively. From each collection site, thirty wild females were set up in the laboratory as separate isofemale lines. At generation F_2 of laboratory culture, mass-bred populations were established by pooling 10 virgin males and females from each isofemale line (600 flies per population) into two 250 mL bottles containing potato-dextrose-agar medium. The populations were expanded and maintained in sizes of at least 1000 individuals at 25°C under 12:12 light:dark cycle for 7-21 generations before transfer to the six thermal regimes (Table S2.1).

2.3.2 Thermal regime experimental design

The experimental populations were initiated at 25°C in bottles containing standard medium (described above) by placing approximately 250 flies per bottle and allowing females to oviposit for two hours prior to removal of all adults. The bottles were then placed into one of six environmental chambers (Panasonic MLR-325H) set to 12:12 light:dark at 16°C, 18°C, 22°C, 25°C, 28°C and 30°C. The developmental temperatures were chosen to represent the range of temperatures that *D. melanogaster* experience in their thermal range permissible to reproduction and development (David *et al.* 1997). Three bottles per population were placed into each cabinet. Oviposition was staggered across several days to synchronise eclosion based on previous assessment of development rates at the different temperatures, thus permitting simultaneous assessment of all population/temperature combinations.

2.3.3 Quantitative trait phenotyping:

2.3.3.1 Fecundity

Daily female fecundity was examined over a 10 day period. The flies were cultured as for the thermal tolerance assays (described below) however imagoes were collected and sorted by sex while still virgin. Thirty pairs of female and male flies from each thermal regime from each population were then placed into individual vials with

medium and mated for 24 hours prior to the commencement of the experiment. Each day the pairs were aspirated into a new vial containing a spoon with blue-dyed medium and 10 µL activated yeast paste (1:3 live yeast:water). The number of eggs each female laid per 24 hour period was recorded. Absolute fecundity was determined to be the mean cumulative number of eggs each female laid.

2.3.3.2 Heat knockdown assay

Heat knockdown time (Hoffmann *et al.* 2002) was used to assess thermotolerance in four-five day old females. Imagoes were collected into mixed-sex cohorts and mated for at least 48 hours. At three-four days post-eclosion, females were separated into groups of 20 using aspiration without CO_2. The females were maintained in 10 dram vials with medium at their respective developmental temperatures prior to the heat assay. Immediately before the assay, the vials were moved to room temperature and individual females were aspirated into 5 mL glass vials, then immersed in a pre-heated water bath set to 38.5°C. Heat knockdown was scored to the nearest second when the fly had become incapacitated. Approximately 30 flies from each population/temperature combination were scored across three replicate assays (blocks), each with approximately 10 flies per population/temperature.

2.3.3.3 Chill coma recovery

We assessed cold tolerance in four-five day old females using a chill-coma recovery assay (Gibert *et al.* 2001). Flies were reared and prepared as for heat knockdown. For the assay, individual females were transferred into empty 1.7 mL Eppendorf tubes and immersed in a pre-chilled 0°C glycol bath and exposed for 6 hours. Flies were then removed and allowed to recover at 25°C, where the time taken (in seconds) for each fly to right itself (stand on its legs) was recorded. Flies that had not recovered at three hours post-stress were excluded from the analysis (four flies). Approximately 30 flies from each population/temperature combination were assessed simultaneously.

2.3.3.4 Egg-to-adult viability

Egg-to-adult viability at each of the six developmental temperatures was determined for each population. At 25°C, approximately 1000 flies were placed onto petri plates containing medium and *ad libitum* yeast paste and females were allowed to oviposit for two hours. Twenty eggs were then transferred into vials containing medium, and

15 vials were set up per population/temperature combination. As progeny eclosed, they were counted and collected into vials containing medium. At four-five days old, the females were frozen and stored at -20°C for the body size measurements (see below).

2.3.3.5 Body size

Wing size was calculated as a proxy for body size (David *et al.* 1997). The right wing from 600 females (50 per population/temperature) was removed using forceps, mounted onto a glass slide with double-sided tape, and secured with a cover slip. Where the right wing could not be mounted, the left was used instead. Each wing was then photographed using a Leica M80 stereo microscope (Leica, Heerbrugg, Switzerland) with a digital camera attached. Eight wing vein landmark positions were obtained (Fig. S2.1) and their x, y coordinates determined using tpsDIG software version 2.17 (Rohlf 2013). Wing area was then measured as centroid size (the square root of the sum of the squared distances from each landmark to the centroid) and calculated using CoordGen8 software (Sheets 2003).

2.3.4 Quantification of transcript abundance

2.3.4.1 Candidate gene rationale

To examine the impact of thermal regime and population of origin on molecular phenotypes from the extremes of the same latitudinal gradient, we chose 18 genes according to the following criteria: 1) involvement in thermal tolerance (*Hsf, Hsrω* and *Hsp70Aa* (Hoffmann *et al.* 2003b)); 2) evidence of population specific expression variation (*Cyp6g1*, *CG9509* and *CG7214* (Hutter *et al.* 2008)), and evidence of GxE for expression (temperature-by-populations interactions; *Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG10910*, *CG30083*, *CG33346*, and *CG42807,* (Levine *et al.* 2011)). Transcripts of interest were chosen based on published literature at the study outset, i.e. Chen *et al.* (2015) and Zhao *et al.* (2015) had not been published but were incorporated into the cross-study comparison *post-*hoc. We examined the multiple isoforms of *Hsf* and *Hsrω* in more detail given the evidence of isoform specific thermal and/or population responsiveness (Cockerell *et al.* 2014; Fujikake *et al.* 2005; Johnson *et al.* 2011; Lakhotia 2011). We designed primers to target a common region of all *Hsf* transcripts as well as 4 isoform specific

primer sets to partition expression of *Hsf-RA*, *RB, RC* and *RD*. The *Hsrω* locus produces multiple nuclear and cytoplasmic long non-coding RNAs, and we examined the longer nuclear transcripts as an isoform subset separately to the shorter cytoplasmic subset. A total of 23 transcripts/transcript subsets were examined in 18 genes. Primer sequences were designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye *et al.* 2012; Table S2.2).

2.3.4.2 Fly collection, mRNA extraction, cDNA synthesis and real-time PCR

The flies for the gene expression assays were collected, sorted by sex and maintained as described for the thermotolerance assays. At day 4-5 post-eclosion, groups of 20 female flies were transferred into 1.7 mL Eppendorf tubes, immediately snap frozen in liquid N_2 and stored at -80°C. Five replicates from each population/temperature combination were collected (60 samples in total).

mRNA was isolated from pools of 20 females per sample using a Dynabeads® mRNA DIRECT™ Purification kit (Life Technologies). Concentrations were determined using the NanoDrop sectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and integrity was assessed using 2% agarose gel electrophoresis. cDNA was synthesised in a 20 µL volume from 50 ng mRNA using a Transcriptor High Fidelity cDNA Synthesis kit (Roche) according to the manufactures' instructions. Two kits were used from separate batches and the samples reverse transcribed from each kit were recorded and incorporated into the statistical analyses. The above steps were performed on small, randomized batches of samples. Real-time PCR was performed in 384-well plates using a *Roche* Lightcycler® 480 and SYBR Green chemistry in a 10 µL reaction. Transcripts were amplified using Lightcycler® 480 SYBR Green 1 master-mix where each well contained 5 µL PCR buffer, 4 µL 1 µM primer mix and 1 µL 1:10 diluted cDNA. Samples were quantified in duplicate (technical replicates), with five biological replicates analysed per population/temperature combination, except for *Cyp6g1* and *Lectin-galC1*which were analysed using three biological replicates. Each biological replicate containing all population/temperature combinations were run together on a plate along with three 'housekeeping' genes, *RpL11, Gapdh2 and CycK*. Each housekeeper was verified for both population and thermo-stability prior to the gene expression assays using two-way analysis of variance (ANOVA; data not shown).

Transcript abundance was quantified relative to the geometric mean (GM) of the housekeepers using the formula: transcript of interest (TOI) = $2^{(GM - TOI)}$.

2.3.5 Reaction norms and performance curves analyses

Fecundity data were analysed as thermal performance curves by assessing BIC of Gaussian functions (Angilletta Jr 2006) fitted to fecundity (F) in the form:

$$\mathrm{F} = \mathrm{a}\exp\left[-0.5\frac{(T-b)^2}{c^2}\right]$$

where a is maximum fecundity (u_{max}), b is optimal temperature (T_{opt}), c is the standard deviation of the mean (performance breadth: T_{br} = 2c) and T denotes a given experimental temperature. Both thermal performance curves and reaction norm functions were fitted using nls() in R (v3.2.0).

For the heat knockdown, chill coma recovery, viability, body size and transcript abundance data population trait means were related to developmental temperature (*i.e.* average population-level reaction norms) by fitting first-to fourth-order polynomial functions. Functions with minimal Bayesian information criterion (BIC; Table S2.3) were selected as best-fitting models (Schwarz 1978).

2.3.6 Statistical Analysis

We next used ANOVA to examine the effects of population and temperature regime on the trait means. Model selection for each trait was determined using diagnostics including Shapiro-Wilk tests for residual normality and Levene's test of equal variances (SAS v9.4, PROC UNIVARIATE and GLM respectively). For the fecundity data, a mixed model was fit with REPEATED/SUBJECT = individual (population) and the GROUP = temperature statements to account for unequal variances driven by temperature differences (PROC MIXED, SAS V9.4). The fecundity data was also analysed using an analysis of covariance (ANCOVA) with population and temperature as fixed factors and wing size as a covariate. Following Klepsatel *et al.* (2013), fecundity thermal performance curve parameters obtained by fitting Gaussian functions were bootstrapped to determine their standard error. To do this, fecundity data were first simulated based on parameter estimates obtained by fitting Gaussian

functions. T_{opt}, u_{max} and T_{br} estimates were then calculated and the process repeated 1000 times for each population.

A fully factorial, two-way general linear-model ANOVA was fit to the heat knockdown and body size data with the fixed effects of population and temperature. A three way fixed-effects general linear model ANOVA was fit separately to each gene/transcript, with the fixed effects of cDNA synthesis kit (kit), population and temperature, and two-way interactions between the three main terms. For all transcripts, the effect of kit was stable across the populations and temperatures therefore the models were reduced to include only the interaction between temperature and population. Both the heat knockdown and gene expression data were log transformed to improve normality. The chill coma recovery data were positively skewed and were analysed using a two-way generalised linear model with gamma distribution (link = log). The egg-to-adult viability data analysed using a generalised linear model (link = logit) to account for bimodal distributions with the fixed effects of population and temperature. For the quantitative traits across all temperatures, pairwise planned contrasts were performed within each population (15 comparisons) and between population comparisons were performed for the six temperatures (6 comparisons) with correction for multiple tests using a false discovery rate (FDR) approach at FDR 0.05 (Benjamini & Hochberg 1995).

Finally, we additionally analysed thermal reaction norms for all quantitative and transcript phenotypes (barring fecundity) using either linear or non-linear regression from the BIC best curve fitting models. For traits with linear reaction norms, linear regression was performed on each population separately with temperature as a continuous factor. For traits with quadratic, cubic or quartic reaction norms, nonlinear regression was performed on the populations separately. Each nonlinear regression had either two, three or four continuous factors (quadratic: temperature and temperature^2; cubic: temperature, temperature^2 and temperature^3; quartic: temperature, temperature^2, temperature^3 and temperature^4). The raw data were fit for each model, and the transcript data were fit with kit as a main factor. The Least squares means (LS means) means derived from the full ANOVA models for all traits are shown for illustrative purposes.

2.4 Results

2.4.1 Effects of thermal regime on quantitative traits

Mean daily fecundity was significantly affected by temperature, population, and the interaction between them (Table 2.1). For each population separately, all pairwise temperature comparisons were performed (15 comparisons), but for interpretative ease between population comparisons were restricted to the same temperature (6 comparisons). Within population pairwise planned contrasts were significant for all comparisons except 25°C *vs* 28°C for both tropical and temperate females (FDR <0.05, Fig. 2.1). On average, the tropical females were significantly more fecund at 16°C, while the temperate females were more fecund at all rearing temperatures except 30°C (FDR <0.05, Fig. 2.1). We also examined the effect of body size on mean daily fecundity using ANCOVA, and found no effect of body size, but significant effects of temperature, population and the interaction between them (temperatature:$F_{5, 346}$ = 1252.5 P <0.0001; population: $F_{1, 346}$ = 7.94, P <0.01; temperature-by-population: $F_{5, 346}$ 23.34 = P <0.0001).

Table 2.1: Results for two-way ANOVAs on the on the fixed effects of developmental temperature, population (temperate and tropical), and the interaction term for fecundity*, heat knock-down time and body size. Significant terms are shown in bold.

Trait	Source of variation	*d.f.*	*SS*	*F*	*P value*
Fecundity	Temperature	5	-	3802.7	**1E-15**
	Population	1	-	7.52	**0.006**
	Temperature × Population	5	-	23.6	**1E-15**
	Error		-		
Heat	Temperature	5	39.832	72.06	**1E-15**
	Population	1	0.421	3.81	**0.052**
	Temperature × Population	5	0.812	1.47	0.199
	Error	344	38.028		
Body size	Temperature	5	14.081	1304.74	**1E-15**
	Population	1	0.432	199.81	**1E-15**
	Temperature × Population	5	0.013	1.25	0.287
	Error	588	1.27		

*Fecundity data were fit using a mixed-model ANOVA to account for unequal variances and uses a likelihood-based estimation where sum of squares (SS) are not output.

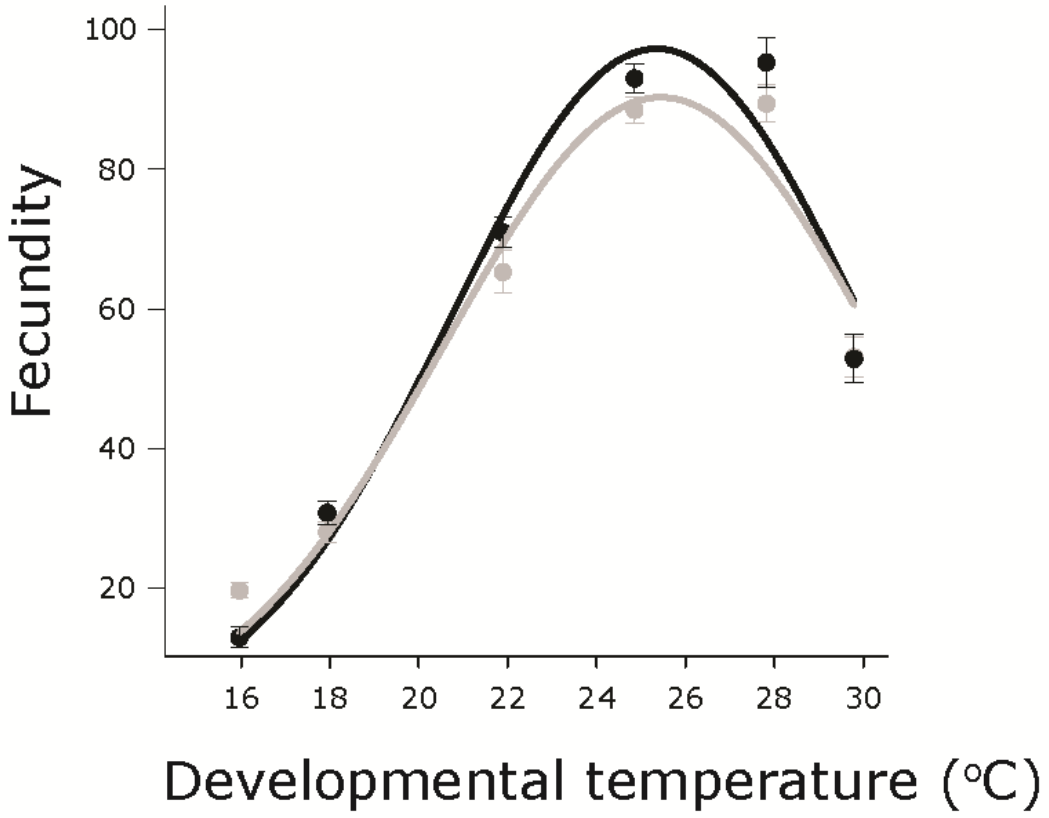

Figure 2.1: Thermal performance curves for average daily fecundity in temperate (black) and tropical (grey) flies. Error bars represent standard error of the least squares mean.

Fecundity performance curve parameter estimate analyses showed that the tropical and temperate females did not differ in their optimal temperatures (T_{opt} temperate: 25.5°C ± 0.12°C; tropical: 25.59°C ± 0.14°C) or performance breadth (T_{br} temperate: 9.33°C ± 0.28°C; tropical: 9.86°C ± 0.32°C). However the populations did differ in their maximum fecundity with temperate females producing more eggs on average per day

than tropical females (u_{max} temperate: 97.29 ± 2.08 eggs/day; tropical: 90.32 ± 1.87 eggs/day).

Rearing temperature significantly affected trait means for heat knockdown and body size (Table 2.1), chill coma recovery and egg-to-adult viability (Table 2.2). Population of origin also significantly impacted mean chill coma recovery time and body size (Tables 2.1, 2.2), and marginally for mean heat knockdown time (Table 2.1, P = 0.0518). The impact of thermal regime on the trait means was similar between the populations, evidenced by the lack of temperature-by-population interactions (Tables 2.1, 2.2) and qualitatively parallel reaction norms (Fig. 2.2: a-d).

Table 2.2: Results for two-way generalised linear model ANOVAs on the fixed effects of developmental temperature, population (temperate and tropical), and the interaction term for chill-coma recovery time and egg-to-adult viability. Significant terms are shown in bold.

Trait	Source of Variation	*d.f.*	Chi-square	*P value*
Cold	Temperature	5	281.3	**1E-15**
	Population	1	17.65	**2.7E-05**
	Temperature × Population	5	7.9	0.162
Viability	Temperature	5	31.88	**6.3E-06**
	Population	1	0.68	0.41
	Temperature × Population	5	7.05	0.217

For heat knockdown, within population pairwise planned contrasts were significant for all comparisons except 16°C *vs* 18°C, 22°C *vs* 25°C and 28°C *vs* 30°C, and the same result was observed in both populations (Fig 2.2a. FDR <0.05 for all other comparisons). Between population contrasts were significant only at 16°C and 18°C due to higher knockdown resistance in temperate females compared to tropical females (Fig. 2.2a. FDR <0.05 and <0.1 respectively). For chill coma recovery; rearing temperature reduced recovery time at the high temperature extremes; within population pairwise planned contrasts were significant for contrasts except 16°C *vs* 18°C, 16°C *vs* 22°C, 16°C *vs* 25°C, 18°C *vs* 25°C and 28°C *vs* 30°C (Fig. 2.2b. FDR <0.05 for all other comparisons). The temperate females were more chill coma resistant than tropical females only at 30°C (FDR <0.05, Fig. 2.2b). Within populations,

body size was significantly different between all temperatures except 16°C *vs* 18°C, and the mean body size of the temperate females was consistently larger than the tropical females across the thermal range (FDR <0.05, Fig. 2.2c). Egg-adult viability was less variable within populations; tropical flies were less viable at 16°C compared to 22°C, 25°C and 28°C, and at 16°C *vs* 25°C for the temperate population, while viability was higher in temperate flies at 18°C *vs* 30°C and 25°C *vs* 30°C (FDR <0.05, Fig. 2.2d). There were no between population differences at each temperature.

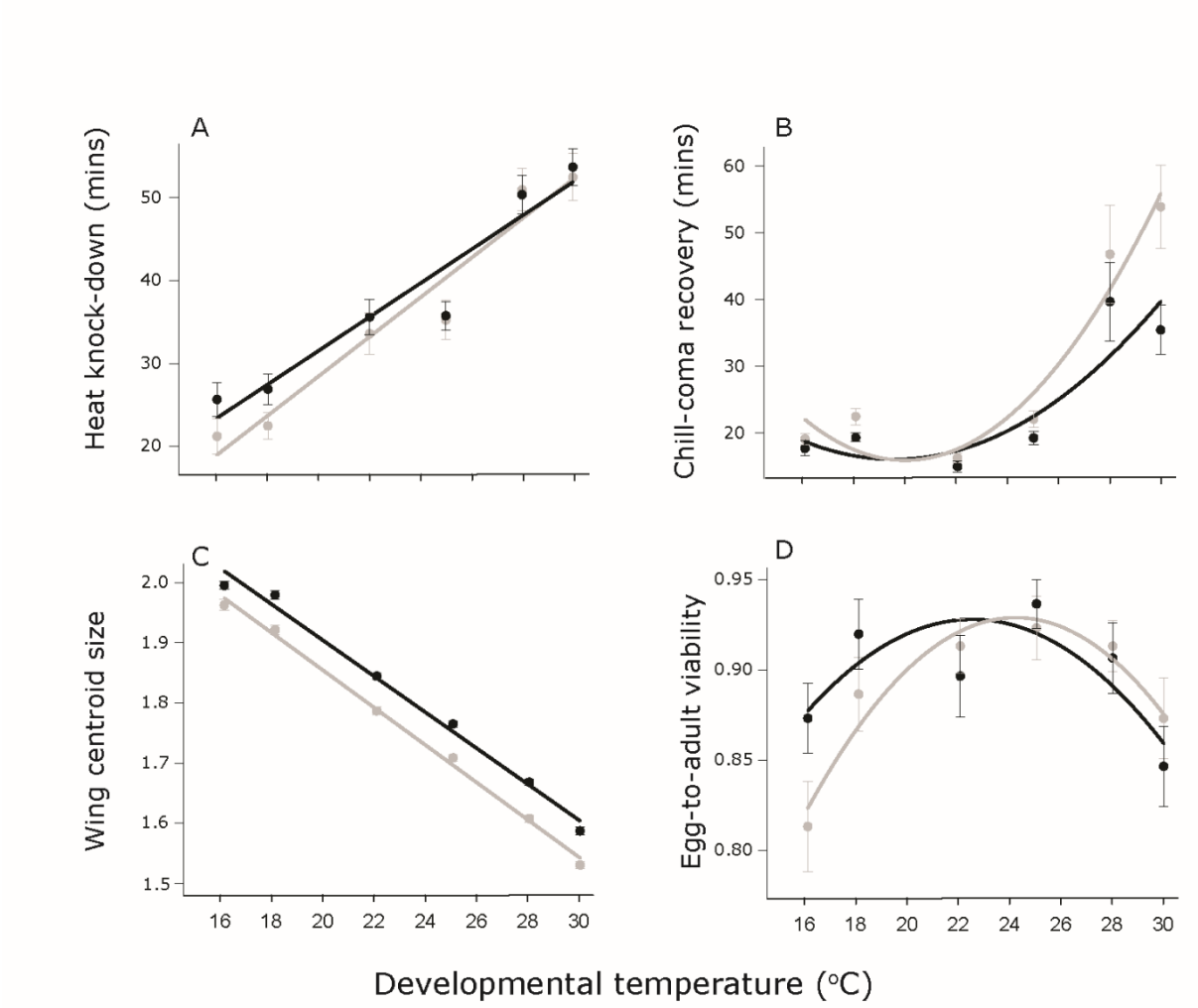

Figure 2.2: Thermal reaction norms for quantitative trait population means in temperate females (black) and tropical (grey) females from eastern Australia. A) Linear reaction norms for heat knockdown resistance, B) quadratic reaction norms for chill coma recovery, C) linear reactions norms for body size (approximated via wing centroid size) and D) quadratic reaction norms for egg-to-adult viability. Error bars represent standard error of the least squares mean.

We also analyzed thermal reaction norms using linear and non-linear multiple regressions. Temperature had a significant positive linear relationship with heat knockdown time in both populations (Table S2.4), where knockdown resistance improved with increasing rearing temperature (Fig. 2.2a). Reaction norms for chill coma recovery in both populations were negative quadratic, with significant main effects of temperature and temperature^2 (Fig. 2.2b, Table S2.4). There was a strong negative linear relationship between temperature and body size, where size decreased with increasing rearing temperature (Fig. 2.2c, Table S2.4). Egg-to-adult viability reaction norms were quadratic, with the parabola concave downwards (i.e. 'bell' shaped, Fig. 2.2d), and although both temperature and temperature^2 main terms were significant, the overall model explained very little of the variation in viability (Table S2.4).

2.4.2 Effects of thermal regime and genotype on transcript-level phenotypes

Similar to the quantitative traits, rearing temperature had the most significant effect on mean transcript expression (22/23 transcript, three-way ANOVA, Table S2.5). Transcript abundance differed between the populations for 15/23 transcripts (Table S2.5), with a bias towards higher mean expression in tropical females (12/15 transcripts, Fig. 2.3). There was little evidence of GxE for expression variation; only two transcripts had significant temperature-by-population interaction terms (*Hsf-RA* and *mag*, P <0.05 and 0.01 respectively, Table S2.5), however these terms did not remain significant following FDR correction. For brevity, we restrict our results to description of reaction norms and not planned contrasts of means within and between populations.

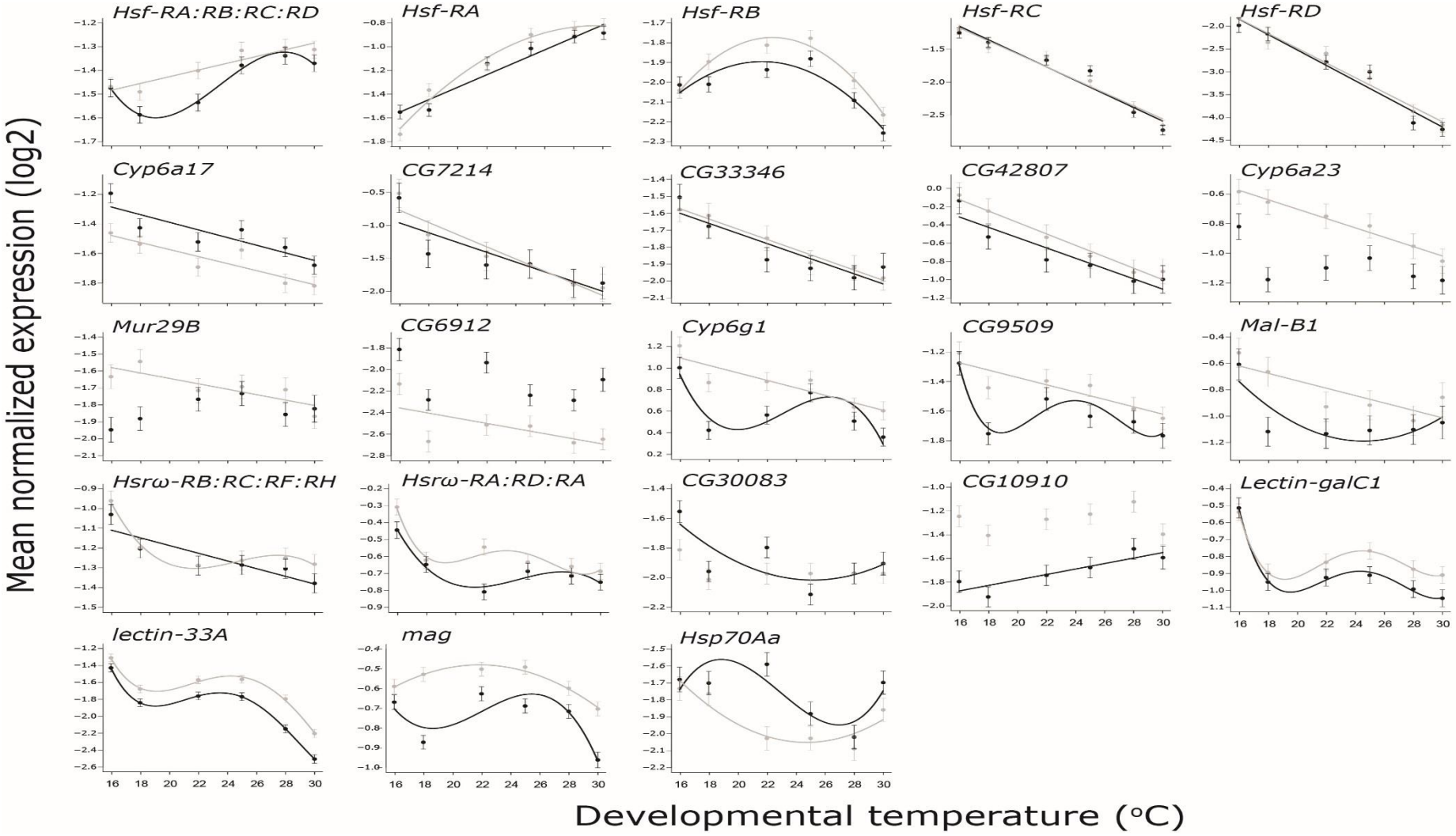

Figure 2.3: Gene expression thermal reaction norms in temperate (black) and tropical (grey) flies for 23 mRNA transcripts (18 genes). Error bars represent standard error of the least squares mean

2.4.2.1 Negative linear expression reaction norms in both populations

We observed an array of gene expression reaction norms fitting to first-fourth order polynomial functions (Table S2.3). For each transcript and population, linear and non-linear multiple regressions were performed with the main terms fit after choosing the order of the reaction norm based on the BIC best fitting models (Fig. 2.3.; Table S2.6). For six transcripts, the main effect of temperature was significant in the linear regression in both the tropical and temperate populations (*Hsf-RC, Hsf-RD, Cyp6a17*, *CG7214*, *CG33346*, *CG42807*, Table S2.6, Fig. 2.3). The populations were invariant in trait means for the latter transcripts except *Cyp6a17*, (ANOVA, Table S2.5) and the reaction norms were negative linear, i.e. expression decreased with increasing rearing temperature (Table S2.6, Fig. 2.3).

2.4.2.2 Population specific, negative linear and non-linear expression reaction norms

For three genes *Cyp6a23*, *Mur29B* and *CG6912*, the expression reaction norms were significantly negative linear in the tropical population only, while the main term of temperature was not significant in the temperate population (Table S2.6, Fig. 2.3). For three genes *Cyp6g1*, *CG9509* and *Mal-B1*, the reaction norms were negative linear in the tropical population and negative quadratic in the temperate population (Table S2.6, Fig. 2.3). Temperature had a significant quadratic relationship with expression (decreasing with slight U shape) in temperate females for *CG30083*, but no main terms were significant in the tropical population (Table S2.6, Fig. 2.3).

The shape of the curves were also differentiated between the two populations for the *Hsrω* transcript subsets; the *Hsrω-RB:RC:RF:RH* temperate population reaction norms were negative linear, while the tropical population curve was sigmoid (s-shaped, decreasing with increasing rearing temperature) with significant temperature, temperature^2 and temperature^3 main terms in the multiple regression (Table S2.6, Fig. 2.3). Both population reaction norms for *Hsω-RA:RD:RG* were negative sigmoid as for *Hsrω-RB:RC:RF:RH*, with an additional temperature^4 component in the multiple regression in the tropical population (Table S2.6, Fig. 2.3).

2.4.2.3 Population specific positive linear and non-linear expression reaction norms

Rearing temperature had a positive linear relationship with expression in both populations for *CG10910*, but was specific to temperate flies for *Hsf-RA* and tropical

flies for *Hsf-RA:RB:RC:RD* (Table S2.6, Fig. 2.3). The tropical population *Hsf-RA* reaction norm was better described by a quadratic rather than linear model (Table S2.6, Fig. 2.3). Temperate expression of *Hsf-RA:RB:RC:RD* was more complex than the tropical population where expression was lowest at 18°C and 22°C and highest at 25°C-30°C, resulting in a positive s-shaped curve with significant linear, quadratic and cubic temperature main terms in the multiple regression (Table S2.6, Fig. 2.3).

2.4.2.4 Higher order non-linear expression reaction norms

For three transcripts, both populations, exhibited similar complex thermal expression curves (*Hsf-RB*, *Lectin-galC1*, *lectin-33A*, Fig. 2.3). The *Hsf-RB* reaction norms were convex curvilinear (i.e. 'bell' shaped), while reaction norms for *Lectin-galC1* and *lectin-33A* were complex, quartic shaped curves where expression was highest and lowest at the low and high temperature extremes respectively, with intermediate expression in the mid-temperature range (Table S2.6, Fig. 2.3). We also observed population specific, non-linear reaction norms for two transcripts, *mag* and *Hsp70Aa*, where the tropical populations exhibited quadratic reaction norms while the temperate populations reaction norms were higher order cubic with significant temperature, temperature^2, and temperature^3 main terms in the multiple regression (Table S2.6, Fig. 2.3).

2.4.2.5 Isoform specific expression reaction norms: Heat shock factor (Hsf)

Interestingly, separate quantification of the four *Hsf* transcript isoforms revealed not only differences in the effects of thermal regime and population on expression means (ANOVA, Table S2.5), but also variation in reaction norms that differed from the 'gene-level' reaction (Fig. 2.3). Quantification of *Hsf* expression at the gene-level (i.e. *Hsf-RA:RB:RC:RD*) would suggest that expression largely increases with rearing temperature with slightly different curve shapes between the populations, and while this is true for one isoform *Hsf-RA*, the *Hsf-RC* and *Hsf-RD* isoform curves were parallel negative linear, while for *Hsf-RB,* both populations exhibited bell shaped reaction norms (Table S2.6, Fig. 2.3).

2.4.3 Cross-study comparisons of transcript thermal response means

Given our evidence based approach in choosing a test set of loci for assessing thermal responses of transcript phenotypes in natural populations of *D. melanogaster* (see Materials and Methods), we next compared our data where possible to that in the literature. For the 18 genes examined here, we documented whether all main terms in the ANOVAs (temperature, population and temperature-by-population interactions) were tested in the other studies, which sex was assessed and genetic background of the populations (inbred or outbred), and where overlap between our study and other studies for a particular term/terms occurred (Table 2.3). As we undertook a candidate gene approach for 18 genes based on previous findings across a range of full transcriptome studies, we could not statistically quantify the degree of overlap but rather qualitatively report common outcomes (Table 2.3).

2.4.3.1 'Core' genes with thermal plasticity and/or geographic variation in diverse *D. melanogaster*

For thermal plasticity, we observed a high degree of overlap with Chen et al., (2015) (15/18 genes, Table 2.3) and Levine et al., (2011) (13/18 genes, Table 2.3) and next with the outbred north American populations (Zhao et al., 2015) (8/18 genes, Table 2.3). A core group of four thermally responsive genes were common to all studies: *Cyp6a23, Mal-B1*, *CG7214*, *CG42807* (Table 2.3). Thermal plasticity of heat shock related genes was observed across studies: *Hsrω* (here, Zhao et al., 2015, and Chen et al., 2015, Table 2.3), *Hsp70Aa* (here and Zhao et al., 2015, Table 2.3) and *Hsf* (here and Chen et al., 2015, Table 2.3).

Table 2.3: Summary of the cross-study comparison for the 18 *D. melanogaster* genes chosen in the current study based on previous evidence of temperature expression plasticity[1,2,3], and/or genotype (geographic) expression variation [1,2,] and/or genotype-by-environment interactions (GxE) between population of origin and thermal regime [1,2,3.] Significant genes* in each study are shown for each category (N/A denotes where terms were not assessed in a study).

Study/design	Thermal plasticity	Genotype/geographic variation	GxE	Inbred (I) or Outbred (O)	Sex
Current study EA‡ Cline ends 16°C, 18°C, 22°C 25°C, 28°C, 30°C	*Hsf, Hsrω, Hsp70Aa*, *Cyp6a17, Cyp6a23, Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912, CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	*Hsf*, *Hsrω*, *Hsp70Aa*, *Cyp6a17, Cyp6a23, Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *Mur29B, CG6912, CG9509*, *CG10910*, *CG30083*	*Cyp6a23**, mag**, Mur29B***	O	Female
Zhao et al.,(2015)[1] NA‡ Cline ends 21°C, 29°C	*Hsrω*, *Hsp70Aa*, *Cyp6a23*, *Lectin-galC1, Mal-B1*, *CG7214*, *CG9509*, *CG42807*	*Cyp6a23*, *Cyp6g1*, *Lectin-galC1*, *Mur29B*, *CG6912*, *CG9509*, *CG42807*	None	O	Male
Levine et al.,(2011)[2] EA‡ Cline ends 18°C, 30°C	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B, CG6912, CG7214, CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B, CG6912, CG10910*, *CG30083*, *CG33346*, *CG42807*	O	Male
Chen et al., (2015)[3] Lab strains 13°C, 18°C, 23°C, 29°C	*Hsf, Hsrω*, *Cyp6a17, Cyp6a23, Cyp6g1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912, CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	N/A	I	Female

**Significance thresholds vary from study to study, we used genes from reported results.* ‡EA = eastern Australia, NA = north America. ***Significant interaction terms in the ANOVA in the current study that were non-significant at FDR 0.05.*

Where we could compare differential expression of genes between populations, we found 7 genes overlapped with the north American populations (Zhao et al., 2015, Table 2.3). Apart from the current study and Levine et al., (2011), only Zhao et al., (2015) tested for GxE but they found no significant interactions for the 18 genes examined here (Table 2.3).

2.4.3.2 Comparison of gene expression reaction norms with other D. melanogaster from the east coast of Australia

The populations studied here, and in Levine et al., (2011), were geographically most comparable: here, females from temperate and tropical populations (southern temperate Melbourne and northern tropical Innisfail) were reared at six temperatures, whereas Levine et al., (2011) compared males from a more southern temperate population (Tasmania) to tropical Innisfail reared at 18 °C and 30 °C. The latter temperatures were chosen with the rationale that they approximate the average 'home' temperatures naturally experienced by flies from the temperate and tropical populations respectively (Levine et al., 2011). The authors reported significant temperature effects on the transcriptome, GxE for 56 genes (FDR 0.1) as well as enrichment of 'home and away' directionality of expression (i.e. higher expression in temperate flies reared at 18°C vs attenuated expression at 30 °C and *vice versa* in the tropical population).

We examined 12 genes exhibiting GxE in Levine et al., (2011), and while temperature significantly impacted all genes and 10 genes were differentially expressed between the populations (Table 2.3), we found little evidence for GxE or 'home and away' gene expression directionality across six rearing temperatures apart from weak signal at the *mag* locus (ANOVA, Table S2.5.). For a more direct comparison, we next analysed trait means only for 18ºC and 30ºC using 3-way ANOVAs. We found less thermal plasticity at 18°C and 30°C compared to Levine et al., (2011) than across our full thermal range, with only 5 genes significant for the main effect of temperature (Table S2.7, Fig. S2.3). The populations differed in transcript abundance for 7 genes, biased to higher expression in the tropical population (Table S2.7, Fig. S2.3). Only two genes, *Cyp6a23* and *Mur29B*, had significant gene-by-environment interaction terms, although the direction of expression was opposite to the 'home and away' pattern observed by Levine et al., (2011) and similar to *mag* for all six temperatures, although

the significance was lost after FDR correction (uncorrected *P* <0.05, FDR <0.2, Table S2.7).

2.4.3.3 Comparison of gene expression reaction norms with inbred D. melanogaster

Chen et al., (2015) classified gene expression reaction norms in inbred female *D. melanogaster* across a broader thermal regime overlapping with the range employed here, (ranging from 13°C to 29°C). Where possible we compared the direction of expression plasticity (i.e. increasing or decreasing with temperature) and reaction norm curvature (Table 2.4). Fifteen genes were comparable between the two studies (Tables 2.3 & 2.4); there was concordance for the direction of expression plasticity (10/15 genes, Table 2.4), with 8/10 genes decreasing in expression with increasing rearing temperature, 1 gene increasing with rearing temperature and 1 gene with U-shaped expression over the thermal regimes (Table 2.4). There was also overlap in reaction norm shape (7/15 genes, Table 2.4) where at least one of the populations here exhibited the same curvature as Chen et al., (2015).

Table 2.4: Cross-study comparison of *D. melanogaster* gene expression reaction norms for 15 genes assessed here (reared at 16°C, 18°C, 22°C 25°C, 28°C, 30°C) with *Chen et al.* (2015) (reared at 13°C, 18°C, 23°C 29°C).

Gene	**Curvature (current study)**		**Curvature (Chen et al., (2015)**	**Plasticity (current study)**	**Plasticity (Chen et al., (2015)**
	Tropical	**Temperate**			
Hsf	linear	quadratic	linear	increasing	increasing
CG33346	linear	linear	linear	decreasing	decreasing
Cyp6a17	linear	linear	quadratic	decreasing	decreasing
CG7214	linear	linear	quadratic	decreasing	decreasing
CG42807	linear	linear	quadratic	decreasing	decreasing
Cypg1	linear	quadratic	linear	decreasing	decreasing
CG9509	linear	quadratic	linear	decreasing	decreasing
Mal-B1	linear	quadratic	linear	decreasing	decreasing
CG6912	linear	-	quadratic	decreasing	decreasing
CG30083	-	quadratic	quadratic	U	U
Cyp6a23	linear	-	quadratic	decreasing	bell
Mur29B	linear	-	quadratic	decreasing	U
CG10910	linear	linear	quadratic	increasing	U
Lectin-33A	quartic	quartic	linear	decreasing	increasing
mag	quadratic	cubic	quadratic	decreasing	bell

2.5 Discussion

We compared thermal plasticity between female *D. melanogaster* from the ends of the eastern Australian temperate-tropical latitudinal gradient for twenty-eight phenotypes across six rearing temperatures. Temperature impacted almost every phenotype ranging from quantitative fitness and morphological traits to gene transcripts. Although there were some differences between the populations for quantitative trait means, fecundity thermal performance and reaction norms for thermotolerance, body size and viability were comparable, supporting a view of ectotherm thermal adaptation by shifts in average trait values rather than reaction norm shape (Fragata *et al.* 2016; Klepsatel *et al.* 2013; Yamahira *et al.* 2007). The gene expression traits revealed more complexity in response curves between the populations, although we found little evidence for a genetic component underpinning the plasticity variation in contrast to previous findings (Levine *et al.* 2011).

2.5.1 Similar performance and reaction norm variation in quantitative traits

For reproductive performance measured as absolute fecundity, we found no difference between tropical and temperate thermal optima or performance breadth, in agreement with cross-continent *D. melanogaster* populations (Klepsatel *et al.* 2013). The temperate females had higher maximum output however, consistent with previous *Drosophila* studies which reject the “hotter is better” hypothesis of performance (Fragata *et al.* 2016; Klepsatel *et al.* 2013). “Hotter is better” predicts a positive correlation in maximal performance with increased thermal selection, i.e. higher output from the tropical population (Angilletta Jr *et al.* 2010). Higher output of temperate flies could result from the positive correlation with body size and fecundity (see Klepsatel *et al.* 2013), but we failed to find a relationship despite the larger size of temperate females across the thermal range. Unlike some *D. subobscura* populations, ‘bigger wasn’t always better’ (Fragata *et al.* 2016); we observed no differences at the highest temperature (30°C), and the tropical females were more fecund than temperate females at the mildest temperature (16°C). The latter result is surprising given that larger more cold adapted flies often perform better at lower temperatures (Bochdanovits & De Jong 2003; Reeve *et al.* 2000).

In contrast to our results, Klepsatel et al., (2013a) found higher fecundity in temperate *D. melanogaster* at intermediate temperatures while tropical females performed better at high temperatures, and in another study measuring reproductive output as productivity, temperate populations did better in the cold but worse in the heat compared to tropical populations (Trotta *et al.* 2006). It is unclear what factors underlie the differences here, possible explanations include insufficient power to detect differences using a narrower, constant thermal range in two populations from the same climatic gradient compared to the six cross-continent populations reared under the fluctuating regime employed by Klepsatel et al., (2013a). On the Australian east coast, while average minimum temperatures decrease with latitude, it is possible that thermal selection at upper temperatures experienced by the populations studied here may be similar given maximum yearly temperatures are largely uniform, and maximum summer temperatures are similar between tropical Innisfail and temperate Melbourne (Hoffmann 2010; Kristensen *et al.* 2015; Overgaard *et al.* 2011). Average temperatures however, do not reflect sudden fluctuations in temperature extremes, which are experienced more frequently by temperate eastern Australian flies compared to their tropical counterparts (Hoffmann 2010). Despite evidence for thermal selection on the Australian east coast in opposing thermotolerance clines (Hoffmann *et al.* 2002; Sgrò *et al.* 2010), we found little variation between the populations for chill coma recovery and heat knockdown; rather the temperate population better resisted heat knockdown at 16°C and 18°C, and recovered from cold exposure faster at 30°C. Maintaining higher stress resistance at the ends of the thermal range could reflect a better ability of temperate flies to withstand temperature extremes. In the laboratory, temperate Australian *D. melanogaster* may be physiologically more capable to withstand sudden, extreme temperature changes than tropical populations (Sgrò *et al.* 2010), although the extent to which natural extremes are mitigated via behavioural avoidance through habitat selection could be important (Feder *et al.* 2000).

Despite variations in mean thermotolerance between the populations, the reaction norms were parallel in shape, consistent with observations of similar thermotolerance plasticity between *D. melanogaster* populations (Bubliy *et al.* 2002; Cooper *et al.* 2012; Hoffmann *et al.* 2005; Hoffmann & Watson 1993) and among widespread and tropical *Drosophila* species (Overgaard *et al.* 2011). We found that rearing temperature impacted the traits in the direction anticipated, i.e. increased resistance to heat

knockdown with increasing rearing temperature and the opposite for chill coma resistance, consistent with high levels of plasticity for stress resistance in response to environmental conditions (discussed in Hoffmann *et al.* 2005). The similarity in phenotypic plasticity at the intra- and –interspecific level holds independent of thermal regimes, (i.e. developmental or short-term acclimation, fluctuating or constant conditions).

For body size, the plastic (i.e. decrease in size with increasing temperature) and genetic responses (consistently larger temperate females) both comprised parallel vertical shifts in the trait means, consistent with most intraspecific comparisons, including continent-wide clinal studies and/or reaction norm analyses (Coyne & Beecham 1987; James *et al.* 1995; Land *et al.* 1999; Trotta *et al.* 2006), but see (Morin *et al.* 1999). We found no population differences in reaction norms for viability, a key indicator of pre-adult fitness, in agreement with European *D. melanogaster* and *D. simulans* populations (Petavy *et al.* 2001). Similarly, thermal plasticity for viability to alternating regimes was invariant between highland *vs* lowland Argentinian populations sampled from opposing latitudinal and altitudinal viability clines (Folguera *et al.* 2008), in contrast however to South American populations that differed slightly in reaction norms, while trait values did not vary over latitude (Land *et al.* 1999). Here however, the impact of temperature was mild, and viability remained above 80% across the thermal range, similar to observations between 14 and 28°C in *D. melanogaster* and *D. simulans* (Petavy *et al.* 2001). Therefore we did not assess viability performance as a trait given our thermal range did not quite capture the stressful temperatures (particularly at the low end) (Kristensen *et al.* 2015; Petavy *et al.* 2001) that might more clearly define performance parameters such as performance breadth.

2.5.2 Expression plasticity

The high degree of thermal plasticity at the transcript level (22/23 transcripts) is unsurprising given our gene selection criteria, with the majority also thermally responsive among different *D. melanogaster* transcriptome studies (Chen *et al.* 2015; Levine *et al.* 2011; Zhao *et al.* 2015). We found four ‘core’ genes with consistent temperature modulated expression independent of genetic background or study

design, suggesting some degree of conserved thermal plasticity in *D. melanogaster*. Chen et al., (2015) reported a higher degree of thermal plasticity than previous estimates from fewer temperatures (Levine *et al.* 2011; Zhao *et al.* 2015; Zhou *et al.* 2012), and for our gene set, we observed the most overlap with Chen et al., (2015) likely due to the broader thermal regimes employed, where plasticity increases with additional environmental exposures.

Although not as extensive as the effect of temperature, mean expression of two-thirds of the genes differed between the tropical and temperate populations, with higher expression levels predominantly biased to the tropical population. Expression variation within and between populations is well documented (Catalán *et al.* 2012; Levine *et al.* 2011; Michalak *et al.* 2007; Müller *et al.* 2011; Oleksiak *et al.* 2002), reflective of ample genetic variation for differential transcript abundance, although the fitness consequences of gene expression divergence remain largely unknown (Evans 2015; Feder & Walser 2005). Expression differences often arise from *cis* regulatory variation, and also copy number variation: here two differentially expressed examples are *Cypa17* and *Cyp6g1*, confirming previous work (Catalán *et al.* 2012; Hutter *et al.* 2008) and copy number variation for these genes are known and related to expression for DDT like pesticide resistance (Schmidt *et al.* 2010) which may vary according latitude along the east Australian coast (Turner *et al.* 2008). Despite the lack of evidence here, there is increasing support for a role of spatially varying selection in maintaining adaptive gene expression variation in diverse environments, where differences in adaptive phenotypes may be evidenced through plasticity (see Levine *et al.* 2011). GxE interactions maintain variation in plasticity across different genotypes and are prevalent in gene expression data (Dayan *et al.* 2015; Levine *et al.* 2011; Zhao *et al.* 2015), although as above, the contribution of gene expression plasticity to organismal fitness is less clear (Hodgins-Davis & Townsend 2009), but general patterns can provide broader insight into evolution in heterogeneous environments.

2.5.3 Lack of support for GxE for expression plasticity

We expanded the developmental thermal regime for 12 highly significant genes from previous research reporting expression GxE at 18°C and 30°C in eastern Australian *D. melanogaster* (Levine *et al.* 2011), but failed to replicate GxE or directional 'home

and away' expression plasticity of expression trait means, even when compared only 18°C and 30°C. One explanation is study design differences; Levine et al., (2011) pooled males from isofemale lines while we assessed mass-bred females derived from isofemale lines which may impact gene expression comparisons owing to patterns of linkage disequilibrium and/or sex-specific effects. Further, while the tropical populations were from the same town in both studies, we assessed a temperate southern mainland population while Levine et al., (2011) compared a temperate population further south from island Tasmania, although Tasmanian *D. melanogaster* are not isolated given the evidence for gene flow between populations (Kennington *et al.* 2003).

Standing genetic variation in different populations will impact GxE; genes that exhibit differential expression for genotypes in different environments are impacted by local but predominantly upstream regulatory sequence variation (Grishkevich & Yanai 2013; Hodgins-Davis & Townsend 2009) and at the genome-wide level may even be categorized as more likely to exhibit GxE by distinctive genomic and structural features (Grishkevich & Yanai 2013). As such, differences in DNA polymorphisms in the temperate populations examined here, and by Levine et al., (2011) compared to tropical Innisfail could be a factor, and we also cannot rule out the impact of seasonal variation on standing genetic variation given the extensive temporal shifts documented in natural *D. melanogaster* (Bergland *et al.* 2014; Itoh *et al.* 2010). Perhaps the highly environmentally plastic nature of the transcriptome (Hodgins-Davis & Townsend 2009) coupled with genetic shifts from sampling season renders replicable signatures of GxE for gene expression traits difficult between temperate and tropical eastern Australian populations.

Although there was little support for genetic variation in mean expression plasticity between the populations, there were differences in the thermal expression reaction norm curves, suggesting a high degree of population-specific plastic variation in contrast to the largely parallel quantitative trait reaction norms. Differences in the shape of the response curves between populations could be passive responses due to neutral sequence variation, thermal stress or other unknown constraints (Gibert *et al.* 1998; Levine *et al.* 2011), or they could be adaptive and therefore useful in identifying putative selection targets (Gibert *et al.* 1998). The results are promising for

future work harnessing the transcriptome as a powerful set of traits and broader thermal regimes to explore the evolutionary basis of plasticity in a modelling framework (e.g. Gibert *et al.* 1998). While the reaction norms were surprisingly dissimilar with previous eastern Australian *D. melanogaster* (Levine et al., 2011), we found repeatability in the direction of plasticity and curvature in at least one of our populations with Chen et al., (2015), highlighting common expression responses to a broader thermal range, but whether the same mechanisms underpin the trait responses remains an open question. It is worth noting that three of the five genes (*Cyp6a23*, *mag* and *Mur29B*) that exhibited different reaction norms between the populations here, and also compared to Chen et al., (2015), exhibited weak evidence for GxE in the current study (either across the entire thermal range or at 18°C and 30°C), suggestive of segregating genetic variation for plasticity in these genes, although more population data is required to further explore this.

2.5.4 Further complexity of transcript reaction norm variation: *Hsf*

Finally, in addition to testing genes with previous population level expression differentiation and/or population-by-temperature interactions, we examined plasticity in three key genes involved in the heat shock response, *Hsp70Aa*, *Hsrω* and the master regulator *Heat shock factor* (*Hsf*). Chen et al., (2015) also reported reaction norms for a number of *Hsps* and similar to here, the genes do not always show a clear relationship with temperature for this thermal range. While we found differences in thermal regulation of the genes, the thermal plasticity appears not to have a discernible genetic component, and in combination with the largely similar thermotolerance phenotypes data suggest a lack of divergence in these populations. Linking heatshock genes to adaptive thermotolerance is problematic however (Telonis-Scott *et al.* 2014), but what was striking here was the complexity of reaction norms for the isoforms of *Hsf* compared to the gene-level reaction norms. Previously, Fujikake et al., (2005) identified alternative isoforms of *Hsf* with two isoforms, *B* and *D* differentially elicited under heat and cold stress respectively, suggesting that in addition to post-transcriptional modifications, transcription of the gene is auto-regulated during thermal stress via alternative splicing. We examined the four isoforms *RA-D* and report three different reaction norms including increasing (*RA*), bell shaped (*RB*) and decreasing (*RC* and *RD*). This speaks of the complexity of the locus but also highlights the

complex nature of transcript level phenotypes where gene isoforms may present as separate traits. Understanding the evolution of traits ultimately depends on how traits are measured here for a gene, and also for quantitative traits; for instance the reaction norms for the final traits of insect size and growth revealed less genetic variation for plasticity than thermal performance curves for growth rate (Kingsolver *et al.* 2004).

2.5.5 Conclusion

In conclusion, we found that populations from tropical and temperate east Australia exhibit similar thermal plasticity for quantitative traits despite evidence for genetic variation for trait values. Our data therefore do not support a model of thermal evolution by plastic shifts but rather in overall trait means. We found no evidence for 'hotter is better' for performance, rather an overall better performance of the temperate population was observed. Our study also incorporated an expanded trait set including a subset of genes exhibiting expression GxE from transcriptome studies, and while there was a higher degree of thermal plasticity for transcript traits, we found little support for a role of genetic variation in maintaining expression plasticity. Instead, we found most overlap in reaction norm shape for expression traits with another study with a similar thermal regime in contrast to studies using fewer exposures. This highlights the need to adequately sample thermal environments when examining the relative contribution of plasticity versus trait mean divergence in populations. Further, the additional complexity in reaction norms between distinct isoforms of the *Hsf* gene demonstrate the importance of trait definition when inferring patterns of plastic and evolutionary responses.

2.5.6 Acknowledgements

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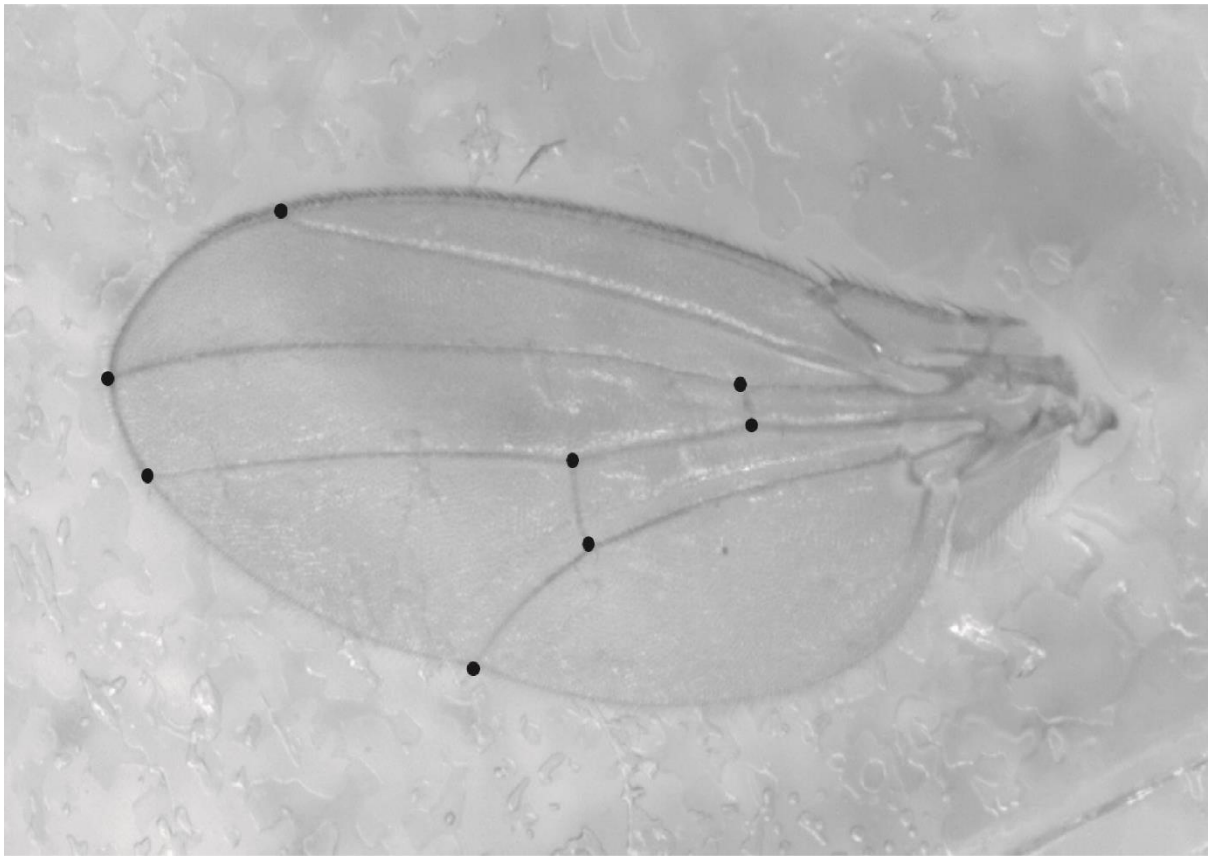
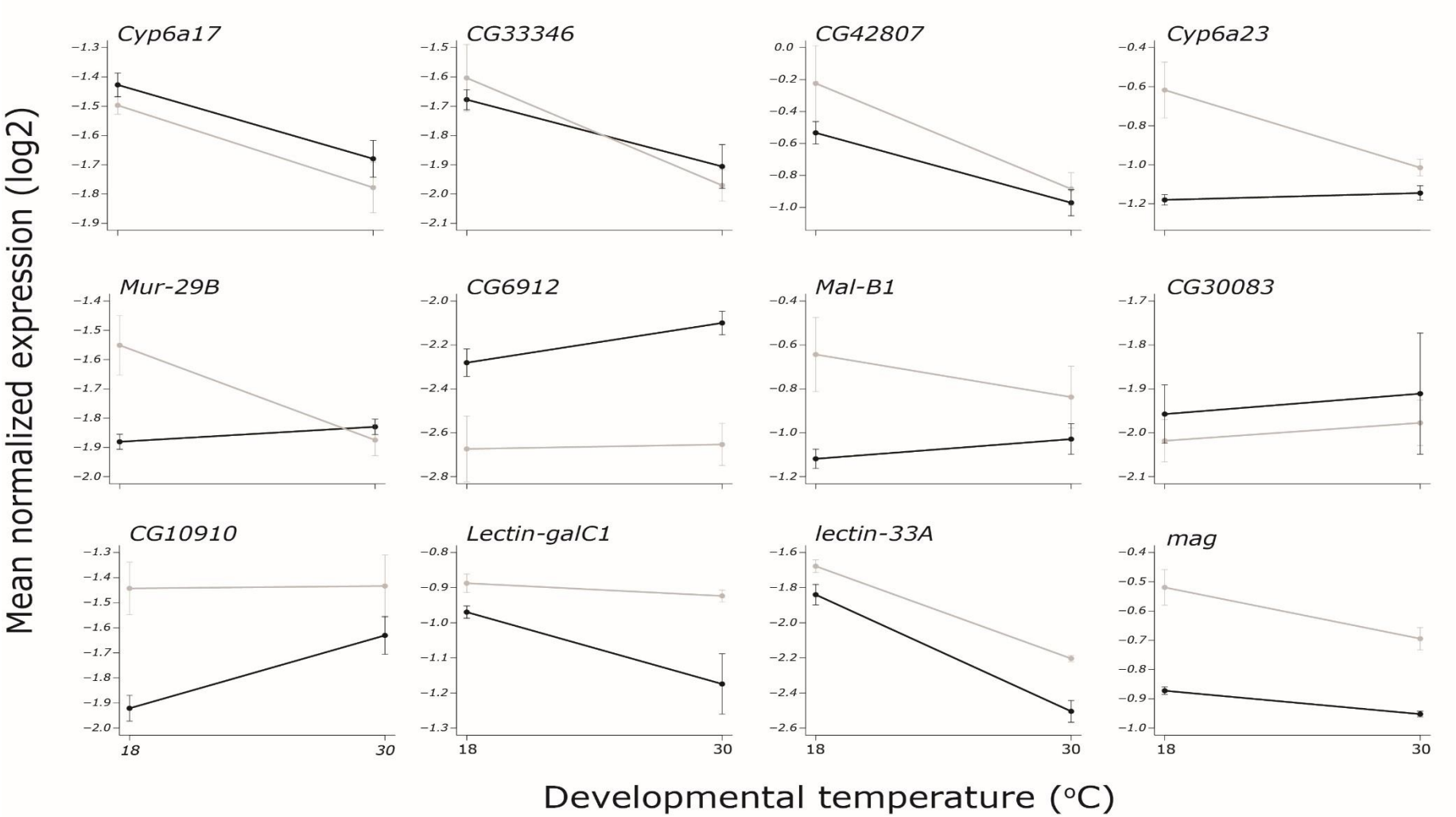
2.6 Supplementary Information



Supplementary Figure 2.1: Wing vein landmark points used to determine wing centroid size. For details see Materials and Methods.



Supplementary Figure 2.2: Thermal developmental reaction norms for temperate (black) and tropical (grey) flies developed at 18°C and 30°C for the twelve transcripts common to this study and Levine *et al.* (2011). Error bars represent standard error of the least squares mean.

Supplementary Table 2.1: Number of generations that each population (Te = Temperate; Tr = Tropical) was maintained in the laboratory at 25^{o}C before each assay was performed.

	16^{o}C		18^{o}C		22^{o}C		25^{o}C		28^{o}C		30^{o}C	
Trait	**Te**	**Tr**	**Te**	**Tr**	**Te**	**Tr**	**Te**	**Tr**	**Te**	**Tr**	**Te**	**Tr**
Heat	11	9	12	10	12	10	12	10	12	10	12	10
Cold	15	13	15	13	15	13	15	13	15	13	15	13
Viability	19	17	19	17	19	17	19	17	19	17	19	17
Wing Size	19	17	19	17	19	17	19	17	19	17	19	17
Fecundity	21	19	21	19	21	19	21	19	21	19	21	19
Transcripts	9	7	9	7	9	7	9	7	9	7	9	7

Supplementary Table 2.2: Primer sequences used for real-time PCR. *Hsf* transcript sequences taken from Fujikake *et al.* (2005).

Gene	Transcript/Subset	Forward primer	Reverse primer
RpL11	*RA:RB*	CGATCCCTCCATCGGTATCT	AACCACTTCATGGCATCCTC
Gapdh2	*RA:RC*	CGTTCATGCCACCACCGCTA	CCACGTCCATCACGCCACAA
CycK	*RA:RB:RC*	CCCAAAAAGAAGCGCTCCAG	AGCTTGCCGGATTTTGGACT
Hsf	*RA:RB:RC:RD*	CAAATTGTGGCGCCTGGTG	AACTTTGGCCATCCTTGGTC
	RA	CTCCTCAGGATGGCCTCAGTTGACG	GTCCATTAGATCAGAGTCGTTAAACAGACCCA
	RB	CCATAATCAAGCGGCTAATTCTTACTGTTGCAG	GTCCATTAGATCAGAGTCGTTAAACAGACCCA
	RC	CTCCTCAGGATGGCCTCAGTTGACG	AATTGGGCGTCCTGCTAAGCGCACC
	RD	CCATAATCAAGCGGCTAATTCTTACTGTTGCAG	AATTGGGCGTCCTGCTAAGCGCACC
Cyp6a17	*RA:RB*	GGTTGTGATGGAAACGCTGC	TCTTCAGGCGAAAAGTCGGT
CG7214	*RA*	AACATGAAGTTCGCCGTTGC	CACATAGGACACACCGGGAG
CG33346	*RB*	CTACGACGCGAGAACCATGA	GGCCGATCTGACTTTAAATTGGT
CG42807	*RA:RB*	GAATGGTTTGGCACAATGCCT	GGCATCACCAATCACCTTGG
Cyp6a23	*RA*	CAGGTCGTCATGGAAACCCT	AGGCGAAAAATCTGTCTGGGT
Mur29B	*RA*	CCGCTTTTGGCTTGGATACG	GGTTGTGATGGAAACGCTGC
CG6912	*RA*	ATTCAGATCGGCCCTGGTTG	ATGTCGGCTTCGCGATGTAT
Cyp6g1	*RA:RB*	CACTCTCGAGAACGGAACCC	TGGGATTGGTCCAGTACTTTGG
CG9509	*RA:RB*	CCCGTCGAATCTGAGTTGC	TGACAGGCCTCATCACTGG
Mal-B1	*RA*	GGCATCACGATTCGGTGAGA	GCCCAACTCCTCGCCATTAT
Hsrω	*RB:RC:RF:RH*	TGCAACCTGCCATACCACTT	TGCGCAGGGTATCGACTTTT
	RA:RD:RG	TAGGAAGCCAGTTGGGCGT	CCGAGTGCGTTTTCAGCA
CG30083	*RB*	GTATGCGGCGGAACTCTCAT	TCCAAGACGAACGGCAAGTA
CG10910	*RB*	TGCTACGTGGATACCGTTCTG	TTCACGGAAAATGCAGGCAC
Lectin-galC1	*RA:RB*	ATCAGCCGGATAATGCTGGG	CTCAAACTTCCGCGAGTCCT
lectin-33A	*RB*	CGTATCTTTGCAGGAGGCCA	TCGAGCACCATTAGTTCGGC
mag	*RA*	CACGATTTGCAACAACGCCT	CATGGAGCTCGCATTGGCAT
Hsp70Aa	*RA*	TCGATGGTACTGACCAAGATGAAGG	GAGTCGTTGAAGTAGGCTGGA

Supplementary Table 2.3: Bayesian Information Criterion (BIC) values for functions fitted to heat tolerance, cold resistance, egg-to-adult viability and transcript abundance data. Significant BICs are bolded.

Trait	Population	Linear	Quadratic	Cubic	Quartic
Heat	Temperate	**1417.884**	1418.782	1423.861	1428.819
	Tropical	**1382.998**	1385.347	1390.344	1395.081
Cold	Temperate	1487.538	**1481.858**	1486.112	1481.409
	Tropical	1570.552	**1553.569**	1558.412	1558.52
Viability	Temperate	-188.0894	**-191.5668**	-187.9377	-186.207
	Tropical	-187.0547	**-196.4115**	-192.0966	-188.966
Hsf RA:RB:RC:RD	Temperate	-43.06261	-40.39166	**-48.20925**	-44.9165
	Tropical	**-64.63253**	-61.74282	-61.7861	-58.824
Hsf RA	Temperate	**-22.38664**	-23.09972	-21.0584	-19.6012
	Tropical	-19.02116	**-32.57406**	-29.52377	-28.677
Hsf RB	Temperate	-22.0709	**-35.12755**	-35.59004	-32.6532
	Tropical	-18.73603	**-63.97743**	-61.72746	-58.3512
Cyp6a17 RA:RB	Temperate	**-24.80936**	-21.40866	-27.13245	-23.9481
	Tropical	**-18.48459**	-15.15017	-13.58123	-10.4325
CG7214 RA	Temperate	**51.02275**	51.06765	50.45003	51.11241
	Tropical	**50.33039**	51.57395	54.49904	57.23447
Cyp6a23 RA	Temperate	**-2.322629**	0.6353737	-1.9247812	-2.86416
	Tropical	**-7.723571**	-4.712124	-1.418036	1.970376
Mur29B RA	Temperate	**-15.04556**	-15.322039	-12.067606	-9.48065
	Tropical	**-15.49695**	-12.802782	-9.567917	-9.39751
CG6912 RA	Temperate	**13.67281**	15.95663	19.23107	10.61728
	Tropical	**13.27312**	15.0405	15.1976	11.69137
Cyp6g1 RA:RB	Temperate	4.917415	7.56265	**2.251229**	1.707621
	Tropical	**-10.97248**	-8.110817	-9.207948	-12.1728
CG9509 RA:RB	Temperate	-0.828424	2.1905279	-1.6243865	**-7.21341**
	Tropical	**-9.085044**	-5.733967	-3.814847	-2.6424
Mal-B1 RA	Temperate	12.47706	**9.527009**	8.208252	8.411453
	Tropical	**18.71257**	18.33617	21.65775	24.90134
Hsrω RB:RC:RF:RH	Temperate	**-26.92904**	-26.04143	-26.30571	-23.0067
	Tropical	-37.32296	-48.13256	**-52.78226**	-49.8649
Hsrω RA:RD:RG	Temperate	-20.64603	-22.84131	**-25.9755**	-22.5744
	Tropical	-37.5186	-38.25178	-39.74853	**-46.436**
CG30083 RB	Temperate	5.128217	**3.008603**	6.09713	4.638043
	Tropical	**-26.67798**	-25.95229	-23.99764	-22.1499
CG10910 RB	Temperate	**-3.230471**	0.1296744	1.6913635	5.030746
	Tropical	**-1.266921**	1.527281	-1.703268	1.604216
Lectin-galC1 RA:RB	Temperate	-19.19359	-18.72298	-27.99307	**-35.3694**
	Tropical	-12.67421	-10.76692	-15.769	**-18.6533**
	Temperate	1.561295	-2.890364	-18.522854	**-23.3253**

lectin-33A RB	Tropical	-8.519447	-12.806249	-47.83732	**-52.0754**
mag RA	Temperate	-24.03927	-29.65024	**-41.3845**	-42.3845
	Tropical	-40.66243	**-52.58094**	-49.63736	-46.2852
Hsp70Aa RA	Temperate	1.68609	5.0306128	**0.4372516**	-2.98123
	Tropical	-14.03673	**-22.84379**	-22.08638	-18.7647

Supplementary Table 2.4: Linear regression (heat resistance and body size) and multiple non-linear regression (Cold resistance and egg-to-adult viability) results for the quantitative traits. Adj. *P* = false discovery rate (FDR) corrected *P*-values. An FDR of 0.05 was used to determine statistical significance.

		Linear component			Quadratic component			Overall model			
Trait	**Population**	**b**	**SE**	***p***	**b**	**SE**	***p***	***df***	***F***	***p***	***Adj. R^2***
Heat	Temperate	2.0311	0.1913	2.20E-16				1, 175	112.700	2.20E-16	0.3882
	Tropical	2.3726	0.1648	2.20E-16				1, 177	207.400	2.20E-16	0.5369
Cold	Temperate	-8.4082	2.9896	0.0055	0.2154	0.0650	0.0011	2, 171	23.610	8.83E-10	0.2072
	Tropical	-15.7278	3.7607	4.62E-05	0.3945	0.0819	3.21E-06	2, 170	39.720	7.04E-15	0.3104
Body size	Temperate	-0.0297	0.0005	2.20E-16				1, 298	3231.000	2.20E-16	0.9153
	Tropical	0.0308	0.0005	2.20E-16				1, 298	2905.000	2.20E-16	0.9067
Viability	Temperate	0.0544	0.0196	0.0068	-0.0012	0.0004	0.0056	2, 28	4.282	0.01684	0.06868
	Tropical	0.0764	0.0191	0.0001	-0.0016	0.0004	0.0003	2, 87	10.390	8.96E-05	0.1743

Supplementary Table 2.5: Three-way fixed-effects general linear-model ANOVA results for transcript abundance for the effects of developmental temperature, population (temperate versus tropical), and the interaction between them*. Adj. *P-value* = false discovery rate (FDR) corrected p-values. An FDR of 0.05 was used to determine statistical significance.

Transcript	Source of variation	*d.f.*	*SS*	*F*	*P-value*	*Adj. P*
Hsf RA:RB:RC:RD	Kit	1	0.358	58.5919	8.39E-10	
	Temperature	5	0.398	13.0441	5.62E-08	1.85E-07
	Population	1	0.064	10.4385	0.002256	0.003991
	Temperature × Population	5	0.025	0.8259	0.537669	0.806789
	Error	47	0.287			
Hsf RA	Kit	1	0.511	34.9559	3.65E-07	
	Temperature	5	5.014	68.5912	2.2E-16	1.27E-15
	Population	1	0.021	1.4523	0.2342	0.283505
	Temperature × Population	5	0.194	2.6576	0.03392	0.39008
	Error	47	0.687			
Hsf RB	Kit	1	0.546	71.3796	5.50E-11	
	Temperature	5	0.933	24.391	5.08E-12	2.34E-11
	Population	1	0.106	13.8063	0.000538	0.001332
	Temperature × Population	5	0.038	0.9967	0.430134	0.806789
	Error	47	0.36			
Hsf RC	Kit	1	0.482	17.1902	0.00014	
	Temperature	5	15.601	111.3319	2.2E-16	1.27E-15
	Population	1	0	0.0101	0.920324	0.920324
	Temperature × Population	5	0.121	0.8605	0.514633	0.806789
	Error	47	1.317			
Hsf RD	Kit	1	0.2	1.8098	0.185	
	Temperature	5	41.37	73.3635	2E-16	1.27E-15
	Population	1	0.06	0.4948	0.4852	0.55798
	Temperature × Population	5	0.39	0.6928	0.6314	0.806789
	Error	47	5.3			
Cyp6a17 RA:RB	Kit	1	0.293	15.6573	0.000255	
	Temperature	5	1.082	11.5469	2.64E-07	5.96E-07
	Population	1	0.467	24.9181	8.65E-06	3.32E-05
	Temperature × Population	5	0.049	0.5264	0.75511	0.868376
	Error	47	0.881			
CG7214 RA	Kit	1	0.002	0.0104	0.919	
	Temperature	5	10.948	9.9097	1.77E-06	2.91E-06
	Population	1	0.055	0.2472	0.6214	0.649645
	Temperature × Population	5	0.214	0.194	0.9633	0.9633
	Error	46	10.164			
CG33346 RB	Kit	1	0.024	0.9289	0.3402	
	Temperature	5	1.326	10.3799	1.05E-06	1.86E-06
	Population	1	0.008	0.3226	0.5728	0.627352
	Temperature × Population	5	0.07	0.5459	0.7405	0.868376

	Error	46	1.176			
CG42807 RA:RB	Kit	1	0.1111	1.2427	0.27075	
	Temperature	5	5.1396	11.4992	3.11E-07	5.96E-07
	Population	1	0.3088	3.4548	0.06947	0.099863
	Temperature × Population	5	0.1087	0.2432	0.94112	0.9633
	Error	46	4.112			
Cyp6a23 RA	Kit	1	0.262	7.8425	0.007437	
	Temperature	5	0.861	5.1476	0.000784	0.000902
	Population	1	1.096	32.7431	7.54E-07	3.47E-06
	Temperature × Population	5	0.235	1.4056	0.240046	0.690132
	Error	46	1.539			
Mur29B RA	Kit	1	0.009	0.3672	0.547508	
	Temperature	5	0.127	1.0457	0.402589	0.402589
	Population	1	0.283	11.7126	0.001313	0.002517
	Temperature × Population	5	0.279	2.3044	0.059753	0.418668
	Error	46	1.113			
CG6912 RA	Kit	1	0.006	0.1375	0.7125	
	Temperature	5	1.618	7.0072	6.11E-05	7.81E-05
	Population	1	2.526	54.6983	2.33E-09	1.34E-08
	Temperature × Population	5	0.174	0.7542	0.5874	0.806789
	Error	46	2.124			
Cyp6g1 RA:RB	Kit	1	0.1508	7.2284	0.01311	
	Temperature	5	1.2874	12.3455	6.84E-06	1.05E-05
	Population	1	0.5011	24.0269	5.95E-05	0.000196
	Temperature × Population	5	0.1116	1.0698	0.40259	0.806789
	Error	23	0.4797			
CG9509 RA:RB	Kit	1	0.043	1.5713	0.216349	
	Temperature	5	1.098	7.9753	1.77E-05	2.54E-05
	Population	1	0.333	12.0824	0.001122	0.002346
	Temperature × Population	5	0.101	0.7315	0.603541	0.806789
	Error	46	1.266			
Mal-B1 RA	Kit	1	0.074	1.2213	0.274847	
	Temperature	5	1.536	5.0711	0.000875	0.000959
	Population	1	0.571	9.4331	0.003572	0.005869
	Temperature × Population	5	0.231	0.7619	0.58194	0.806789
	Error	46	2.786			
Hsrω RB:RC:RF:RH	Kit	1	0.972	84.6087	4.42E-12	
	Temperature	5	0.655	11.4074	3.07E-07	5.96E-07
	Population	1	0.026	2.268	0.1388	0.177356
	Temperature × Population	5	0.018	0.3121	0.9032	0.9633
	Error	47	0.54			
Hsrω RA:RD:RG	Kit	1	0.5598	51.1845	4.77E-09	
	Temperature	5	0.6928	12.6703	8.2E-08	2.36E-07
	Population	1	0.149	13.6265	0.000579	0.001332
	Temperature × Population	5	0.0957	1.7502	0.141741	0.465721
	Error	47	0.514			
CG30083 RB	Kit	1	0.007	0.313	0.578567	
	Temperature	5	0.685	5.8231	0.000302	0.000365

	Population	1	0.069	2.9371	0.093298	0.126227
	Temperature × Population	5	0.238	2.0211	0.093277	0.429074
	Error	46	1.082			
CG10910 RB	Kit	1	0.266	7.2728	0.00975	
	Temperature	5	0.619	3.383	0.01099	0.01149
	Population	1	2.667	72.8189	4.9E-11	5.64E-10
	Temperature × Population	5	0.183	0.9996	0.42871	0.806789
	Error	46	1.685			
Lectin-galC1 RA:RB	Kit	1	0.0275	3.7168	0.066304	
	Temperature	5	0.681	18.4066	2.3E-07	5.88E-07
	Population	1	0.0648	8.7644	0.007011	0.01075
	Temperature × Population	5	0.0263	0.7096	0.622305	0.806789
	Error	23	0.1702			
lectin-33A RB	Kit	1	0	0.0001	0.9908	
	Temperature	5	4.674	91.3204	2.2E-16	1.27E-15
	Population	1	0.706	68.9641	1.06E-10	8.13E-10
	Temperature × Population	5	0.091	1.7867	0.1344	0.465721
	Error	46	0.471			
mag RA	Kit	1	0.0142	2.335	0.13334	
	Temperature	5	0.4127	13.5365	3.95E-08	1.51E-07
	Population	1	0.4993	81.8783	8.86E-12	2.04E-10
	Temperature × Population	5	0.1203	3.9464	0.00463	0.10649
	Error	46	0.2805			
Hsp70Aa RA	Kit	1	0.215	9.189	0.003953	
	Temperature	5	0.916	7.817	2.03E-05	2.75E-05
	Population	1	0.356	15.1976	0.000306	0.00088
	Temperature × Population	5	0.255	2.1753	0.072812	0.418668
	Error	47	1.102			

*See materials and methods for explanation of kit term.

Supplementary Table 2.1: Multiple linear and non-linear regression for transcript abundance data. Adj. *P* = false discovery rate (FDR) corrected p-values. An FDR of <0.05 was used to determine statistical significance. See Materials and Methods for explanation of kit component.

		Kit component			Linear component			Quadratic component			Cubic component			Quartic component			Overall model				
Transcript	**Population**	**b**	**SE**	***p***	**b**	**SE**	***p***	**b**	**SE**	***p***	**b**	**SE**	***p***	**b**	**SE**	***p***	***df***	***F***	***p***	***Adj. p***	***Adj. R^2***
Hsf RA:RB:RC:RD	Temperate	-0.2229	0.0499	0.0002	-1.2777	0.3744	0.0022	0.0563	0.0165	0.0025	-0.0008	0.0002	0.0025	-	-	-	4, 25	18.42	3.64E-07	1.2E-06	0.7061
	Tropical	-0.2006	0.0300	2.310E-07	0.0144	0.0025	4.470E-06	-	-	-	-	-	-	-	-	-	2, 27	43.28	3.79E-09	1.58E-08	0.7446
Hsf RA	Temperate	-0.2892	0.0733	0.0005	0.0521	0.0054	3.190E-10	-	-	-	-	-	-	-	-	-	2, 27	77	7.00E-12	4.03E-11	0.8398
	Tropical	-0.3205	0.0495	7.410E-07	1.7168	0.1141	2.410E-14	-0.5083	0.1144	0.0001	-	-	-	-	-	-	3, 26	100.1	2.11E-14	9.02E-13	0.9111
Hsf RB	Temperate	-0.2490	0.064178	0.0006	0.2239	0.0550	0.0004	-0.0051	0.0012	0.0002	-	-	-	-	-	-	3, 26	21.52	3.23E-07	1.14E-06	0.6797
	Tropical	-0.2883	0.0294	3.070E-10	-0.2084	0.0676	0.0044	-0.6963	0.0678	1.200E-10	-	-	-	-	-	-	3, 26	62.73	4.90E-12	3.56E-11	0.8646
Hsf RC Melbourne	Temperate	-0.3636	0.1110	0.0029	-0.1057	0.0082	4.650E-13	-	-	-	-	-	-	-	-	-	2, 27	84.13	2.51E-12	2.89E-11	0.8515
	Tropical	-0.2161	0.0774	0.0095	-0.0986	0.0065	9.450E-15	-	-	-	-	-	-	-	-	-	2, 27	116.6	5.19E-14	9.02E-13	0.8886
Hsf RD	Temperate	-0.2788	0.1900	0.1538	-0.1721	0.0140	1.510E-12	-	-	-	-	-	-	-	-	-	2, 27	79.8	4.64E-12	3.56E-11	0.8446
	Tropical	-0.1071	0.1533	0.4907	-0.1611	0.0128	8.920E-13	-	-	-	-	-	-	-	-	-	2, 27	78.74	5.41E-12	3.56E-11	0.8428
Cyp6a17 RA:RB	Temperate	-0.1041	0.7036	0.1506	-0.0233	0.0052	0.0001	-	-	-	-	-	-	-	-	-	2, 27	10.04	0.00055	0.000936	0.384
	Tropical	-0.2544	0.0647	0.0005	-0.0241	0.0054	0.0001	-	-	-	-	-	-	-	-	-	2, 27	16.31	2.27E-05	5.5E-05	0.5136
CG7214 RA	Temperate	-0.0179	0.3074	0.9540	-0.0755	0.0213	0.0015	-	-	-	-	-	-	-	-	-	2, 26	8.418	0.001517	0.002326	0.3464
	Tropical	0.1582	0.2037	0.4440	-0.0914	0.0171	1.100E-05	-	-	-	-	-	-	-	-	-	2, 27	15.06	4.04E-05	9.29E-05	0.4924
CG33346 RB	Temperate	0.0089	0.1020	0.9314	-0.0283	0.0071	0.0005	-	-	-	-	-	-	-	-	-	2, 26	11.19	3.11E-04	0.00055	0.4213
	Tropical	-0.0501	0.0669	0.4600	-0.0301	0.0056	1.120E-05	-	-	-	-	-	-	-	-	-	2, 27	14.49	5.31E-05	0.000116	0.482
CG42807 RA:RB	Temperate	-0.0465	0.1780	0.7961	-0.0546	0.0124	0.0002	-	-	-	-	-	-	-	-	-	2, 26	12.54	1.54E-04	0.000295	0.4519
	Tropical	-0.1173	0.1304	0.3762	-0.0627	0.0109	4.260E-06	-	-	-	-	-	-	-	-	-	2, 27	16.54	2.04E-05	5.21E-05	0.5173
Cyp6a23 RA	Temperate	-0.1436	0.1225	0.2519	-0.0130	0.0085	0.1371	-	-	-	-	-	-	-	-	-	2, 26	1.274	0.2965	0.303089	0.01921
	Tropical	-0.1688	0.0774	0.0381	-0.0315	0.0065	4.570E-05	-	-	-	-	-	-	-	-	-	2, 27	13.37	9.23E-05	0.000193	0.4603
Mur29B RA	Temperate	-0.0215	0.0984	0.8290	0.0057	0.0068	0.4150	-	-	-	-	-	-	-	-	-	2, 26	0.6287	0.5412	0.5412	0
	Tropical	0.0241	0.0680	0.7258	-0.0161	0.0057	0.0088	-	-	-	-	-	-	-	-	-	2, 27	4.156	0.02651	0.031268	0.1792
CG6912 RA	Temperate	0.0671	0.1614	0.6810	-0.0157	0.0112	0.1720	-	-	-	-	-	-	-	-	-	2, 26	1.88	0.1728	0.180655	0.05911
	Tropical	0.1394	0.1099	0.2152	-0.0232	0.0092	0.0179	-	-	-	-	-	-	-	-	-	2, 27	4.267	0.02453	0.029694	0.1839

Cyp6g1 RA:RB	Temperate	-	-	-	-3.0872	1.1073	0.0145	0.1377	0.0490	0.0139	-0.0020	0.0007	0.0130	-	-	-	3, 14	3.93	0.03157	0.03542	0.3408
	Tropical	-	-	-	-0.0347	0.0069	0.0001	-	-	-	-	-	-	-	-	-	1, 16	25.01	0.000131	0.000261	0.5855
CG9509 RA:RB	Temperate	-0.1994	0.1130	0.0909	-17.5800	5.3630	0.0033	1.1300	0.3593	0.0045	-0.0318	0.0106	0.0061	0.0003	0.0001	0.0084	5, 23	5.344	0.002107	0.003127	0.4368
	Tropical	0.0049	0.0757	0.9488	-0.0242	0.0063	0.0007	-	-	-	-	-	-	-	-	-	2, 27	7.356	0.002818	0.004051	0.3048
Mal-B1 RA	Temperate	-0.0803	0.1577	0.6151	-0.2999	0.1161	0.0160	0.0061	0.0025	0.0208	-	-	-	-	-	-	3, 35	3.478	0.03084	0.03542	0.2098
	Tropical	-0.1372	0.1203	0.2640	-0.0284	0.0101	0.0090	-	-	-	-	-	-	-	-	-	2, 27	4.379	0.02253	0.028788	0.189
Hsrω RB:RC:RF:RH	Temperate	-0.3684	0.0679	9.770E-06	-0.0195	0.0050	0.0006	-	-	-	-	-	-	-	-	-	2, 27	16.96	1.69E-05	4.57E-05	0.524
	Tropical	-0.3244	0.0355	1.870E-09	-0.0451	0.0786	5.610E-06	0.3437	0.0787	0.0002	-0.2259	0.0814	0.0103	-	-	-	4, 25	34.42	7.87E-10	3.62E-09	0.8218
Hsrω RA:RD:RG	Temperate	-0.3140	0.0723	0.0002	-1.4985	0.5424	0.0106	0.0622	0.0239	0.0153	-0.0008	0.0003	0.0208	-	-	-	4, 25	6.811	0.00075	0.001189	0.4449
	Tropical	-0.2347	0.0382	2.350E-06	-0.5314	0.0842	1.600E-06	0.1953	0.0844	0.0295	-0.2194	0.0873	0.0191	0.2609	0.0842	0.0049	5, 24	17.6	2.45E-07	9.39E-07	0.741
CG30083 RB	Temperate	-0.0565	0.1410	0.9621	-0.2557	0.1037	0.0209	0.0051	0.0022	0.0313	-	-	-	-	-	-	3, 35	4.691	0.009858	0.012956	0.2834
	Tropical	0.1113	0.0564	0.0591	-0.0056	0.0047	0.2464	-	-	-	-	-	-	-	-	-	2, 27	2.851	0.07526	0.080511	0.1132
CG10910 RB	Temperate	0.3433	0.1206	0.0085	0.0286	0.0084	0.0021	-	-	-	-	-	-	-	-	-	2, 26	6.624	0.004733	0.006403	0.2866
	Tropical	0.2514	0.0862	0.0071	0.0033	0.0072	0.6495	-	-	-	-	-	-	-	-	-	2, 27	4.276	0.02437	0.029694	0.1843
Lectin-galC1 RA:RB	Temperate	-	-	-	-10.6800	2.8140	0.0022	0.6740	0.1887	0.0034	-0.0186	-0.0055	0.0051	0.0002	0.0001	0.0075	4, 13	23.27	7.95E-06	2.44E-05	0.8398
	Tropical	-	-	-	-11.7800	4.4760	0.0207	0.7470	0.3002	0.0272	-0.0207	0.0088	0.0352	0.0002	0.0001	0.0450	4, 13	6.661	0.003823	0.005329	0.5712
Lectin-33A RB	Temperate	0.0402	0.0856	0.6428	-13.7600	4.0620	0.0025	0.8666	0.2722	0.0041	-0.0237	0.0080	0.0069	0.0002	0.0001	0.0118	5, 23	42.02	8.04E-11	4.11E-10	0.8799
	Tropical	-0.0040	0.0347	0.9083	-1.2143	0.0767	3.280E-14	-0.4608	0.0768	3.430E-06	-0.7179	0.0795	3.450E-09	0.2022	0.0767	0.0144	5, 24	76.68	5.88E-14	9.02E-13	0.9288
magro RA Melbourne	Temperate	-0.0249	0.0648	0.7037	-1.5420	0.4253	0.0013	0.0719	0.0187	0.0008	-0.0011	0.0003	0.0005	-	-	-	4, 24	8.597	1.88E-04	0.000346	0.5205
	Tropical	-0.0525	0.0355	0.1515	-0.1936	0.0818	0.0256	-0.3411	0.0820	0.0003	-	-	-	-	-	-	3, 26	7.838	0.000691	0.001135	0.4143
Hsp70Aa RA	Temperate	-0.1163	0.1123	0.3103	2.2760	0.8423	0.0122	-0.1019	0.0371	0.0110	0.0015	0.0005	0.0106	-	-	-	4, 25	2.833	0.0458	0.050162	0.2018
	Tropical	-0.2367	0.0583	0.0004	-0.4841	0.1342	0.0013	0.4861	0.1345	0.0013	-	-	-	-	-	-	3, 26	14.6	8.98E-06	2.58E-05	0.5846

Supplementary Table 2.2: Three-way fixed-effects general linear-model ANOVA results for transcript abundance showing the effects of developmental temperature, population, and the interaction between them for temperate and tropical flies developed at 18ºC and 30ºC for the twelve transcripts common to this study and Levine *et al.* (2011). Adj. *P* = false discovery rate (FDR) corrected *P*-values. An FDR of <0.05 was used to determine statistical significance.

Transcript	Source of variation	*d.f.*	*SS*	*F*	*P*	*Adj. P*
Cyp6a17 RA:RB	Kit	1	0.007	0.3943	0.53949	
	Temperature	1	0.356	19.6203	0.000487	0.002924
	Population	1	0.042	2.3281	0.03242	0.19452
	Temperature × Population	1	0.001	0.0558	0.816389	0.890606
	Error	15	0.272			
CG33346 RB	Kit	1	0.019	0.6795	0.423592	
	Temperature	1	0.436	15.5585	0.001468	0.005872
	Population	1	0.001	0.0183	0.894396	0.894396
	Temperature × Population	1	0.013	0.4626	0.507509	0.672173
	Error	14	0.392			
CG42807 RA:RB	Kit	1	0.0325	0.3108	0.586018	
	Temperature	1	1.4436	13.7923	0.002313	0.006939
	Population	1	0.1407	1.3439	0.265739	0.318887
	Temperature × Population	1	0.0373	0.3562	0.560144	0.672173
	Error	14	1.4653			
Cyp6a23 RA	Kit	1	0.0094	0.2812	0.604243	
	Temperature	1	0.1639	4.9211	0.043573	0.087146
	Population	1	0.5008	15.0325	0.001675	0.005025
	Temperature × Population	1	0.1878	5.6376	0.03242	0.19452
	Error	14	0.4664			
Mur29B RA	Kit	1	0.007	0.3446	0.56653	
	Temperature	1	0.072	3.6344	0.07733	0.132566
	Population	1	0.102	5.1626	0.03937	0.059055
	Temperature × Population	1	0.172	8.7002	0.01055	0.1266
	Error	14	0.277			
CG6912 RA	Kit	1	0.011	0.2021	0.659951	
	Temperature	1	0.035	0.6736	0.42555	0.51066
	Population	1	1.044	19.9041	0.000538	0.00215
	Temperature × Population	1	0.021	0.4014	0.536591	0.672173
	Error	14	0.734			
Mal-B1 RA	Kit	1	0.0351	0.4775	0.50084	
	Temperature	1	0.0238	0.3232	0.57869	0.6062
	Population	1	0.4335	5.8935	0.02927	0.050177
	Temperature × Population	1	0.0649	0.8827	0.3634	0.622971
	Error	14	1.0297			
CG30083 RB	Kit	1	0	0.0008	0.9773	
	Temperature	1	0.008	0.2781	0.6062	0.6062
	Population	1	0.018	0.6162	0.4455	0.486

	Temperature × Population	1	0	0.0008	0.9777	0.9777
	Error	14	0.416			
CG10910 RB	Kit	1	0.035	0.8091	0.383605	
	Temperature	1	0.13	2.9703	0.106811	0.155867
	Population	1	0.571	13.0374	0.002837	0.006809
	Temperature × Population	1	0.116	2.6496	0.125867	0.503468
	Error	14	0.613			
Lectin-galC1 RA:RB	Temperature	1	0.0093	3.0885	0.1169	0.155867
	Population	1	0.028	9.3094	0.01579	0.03158
	Temperature × Population	1	0.005	1.653	0.23452	0.562848
	Error	8	0.0241			
lectin-33A RB	Kit	1	0.005	0.527	0.479823	
	Temperature	1	1.53	152.7786	6.39E-09	7.67E-08
	Population	1	0.255	25.4779	0.000178	0.001069
	Temperature × Population	1	0.017	1.6572	0.218862	0.562848
	Error	14	0.14			
mag RA	Kit	1	0.0005	0.0628	0.805775	
	Temperature	1	0.075	9.8808	0.007186	0.017246
	Population	1	0.4076	53.7123	3.74E-06	4.49E-05
	Temperature × Population	1	0.0091	1.1941	0.292943	0.585886
	Error	14	0.1062			

Declaration for Thesis Chapter 3

"Hardening but not population divergence impacts temporal expression profiles of candidate genes for desiccation resistance in Drosophila melanogaster"

This thesis chapter is in publication format for submission

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Contribution (%)
Experimental design, execution of experimental work, data analysis, manuscript writing	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of Contribution	Contribution (%)
Carla M. Sgro	Experimental design, manuscript writing	15%
Marina Telonis-Scott	Experimental design, manuscript writing	15%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	**Date** 19/12/2016
Main Supervisor's Signature	**Date** 19/12/2016

CHAPTER 3

Hardening but not population divergence impacts temporal expression profiles of candidate genes for desiccation resistance in Drosophila melanogaster

3.1 ABSTRACT

Research surrounding climate change has recently recognised the need to focus not only on the impact of increasing temperature, but on other abiotic factors such as water availability. In environments where inconsistent fluctuation in water occurs, organisms can use phenotypic plasticity to counteract negative effects, increase their desiccation resistance and maintain their optimal phenotype. Despite evidence for desiccation plasticity in geographically distinct *Drosophila melanogaster* populations along the Australian east coast, the molecular basis of this trait is still poorly understood. I therefore assessed the temporal expression of twelve candidate genes for desiccation resistance prior to, during and following desiccation stress and characterised their response to plasticity in a temperate and tropical population of *D. melanogaster*. I also examined the whole animal, phenotypic response to desiccation stress. I found that all candidate genes responded to desiccation stress with ten of the twelve also exhibiting a plastic response. I also found strong evidence of geographic variation in phenotypic plasticity in desiccation resistance, with each population mounting markedly different plastic responses. Despite this, population differences were not replicated at the molecular level in the candidate genes linked to desiccation resistance. Unlike the well-characterised heat shock response, I found that much of the changes in transcript abundance occurred during desiccation stress rather than in the hours and days following exposure to the stress. Given the complex physiological nature of desiccation resistance and the tissue-specificity of many of the candidate genes, my results provide the first insights into the molecular basis of desiccation plasticity.

3.2 INTRODUCTION

Water availability impacts the behaviour, abundance and range distribution of terrestrial ectotherms (Chown & Nicolson 2004; Chown *et al.* 2011). For small insects highly sensitive to environmental change like *Drosophila*, spatial distribution is limited by genetic variation for desiccation resistance, where more resistant species and populations inhabit drier habitats compared to their less resistant counterparts (Kellermann *et al.* 2009; Parsons 1982; van Herrewege & David 1997). The physiological adaptations underpinning the desiccation resistance spectrum can arise through multiple mechanisms including increased water retention, bulk water storage, and greater water loss tolerance (Gibbs *et al.* 2003; Gibbs & Matzkin 2001). Water budgeting mainly occurs via the epicuticle barrier, through respiratory control across the spiracles or gut epithelia and by excretion and osmoregulation via the Malpighian tubules (MTs) (Hadley 1994). Intra- and inter-specific data in *Drosophila* demonstrate that desiccation resistance is achieved by remarkably diverse, genotype-specific combinations of the different water balance mechanisms (Chippindale *et al.* 1998; Folk *et al.* 2001; Gibbs & Matzkin 2001; Marron *et al.* 2003; Telonis-Scott *et al.* 2006).

The environment also impacts patterns of desiccation resistance; female *D. melanogaster* increased desiccation resistance following a non-lethal exposure to desiccation ('hardening') (Hoffmann 1990; Hoffmann 1991). Flexibility to improve desiccation resistance via phenotypically plastic responses such as hardening is of interest to biologists studying insects in a changing climate, although the contribution of phenotypic plasticity to fitness under naturally fluctuating humidity remains unclear. In the laboratory plastic resistance physiology appears to be as variable as genetic resistance, although there may be some shared mechanisms (Hoffmann 1990). Hardening may cause changes in cuticular permeability to stem water loss in *D. melanogaster* (Bazinet *et al.* 2010), including changes in cuticular hydrocarbon abundances (Stinziano *et al.* 2015). Fixed cuticle structure may also influence hardening where melanic species of four Indian *Drosophila* subgenera exhibited hardening responses despite lack of changes in cuticular traits or cuticular water loss, while non-melanic species lacked resistance plasticity (Kalra *et al.* 2014). Alternatively, Parkash *et al.* (2014) associated increases in desiccation resistance in hardened *D.*

melanogaster to changes in osmolyte concentration, owing to increases in bulk water storage.

Despite the well characterised physiology of desiccation resistance, key knowledge gaps remain in linking the desiccation resistant/susceptible phenotype to the underlying molecular mechanisms, and it is unknown if genetic and plastic tolerances share similar molecular architectures or present as separate traits. Candidate genes for desiccation resistance to date derive from approaches focussed on either gene/s of large effect or from complex trait analyses, and span the range of water balance mechanisms and other plausible functions including initial hygrosensing and activation of stress response pathways, stress sensing and excretory/osmoregulatory balance in the gut and Malpighian tubules (MTs), insulin signalling, the cuticular water loss barrier (CHCs) and tissue protectants including trehalose (reviewed in Chown *et al.* 2011; Telonis-Scott *et al.* 2016). Surprisingly few of these genes have been examined in detail in natural *Drosophila* varying in desiccation resistance, and none in the context of hardening. To address this, I examined patterns of genetic (basal) desiccation resistance and hardening (plastic) responses to desiccation stress in tropical and temperate *D. melanogaster* females and profiled the expression of key candidate genes with and without a non-lethal hardening pre-exposure over a comprehensive stress/recovery time-course.

Given previous evidence for plasticity and genetic differentiation for desiccation resistance in tropical and temperate *D. melanogaster* and *D. simulans* populations (Hoffmann, 1991), I took advantage of the 'cline-end' sampling strategy (e.g. Hoffmann & Watson 1993; Levine *et al.* 2011; Morin *et al.* 1999; Trotta *et al.* 2006) and examined desiccation plasticity in a tropical and temperate population from eastern Australia. I profiled candidate genes linked either directly or indirectly to desiccation resistance spanning a range of potential desiccation and defence mechanisms. Desiccation specific genes included fluid transporting diuretic neuropeptides *Capa*, *CapaR*, *and klu* (Kean *et al.* 2002; Terhzaz *et al.* 2014) and antidiuretic peptides *ITP* and *sNPF (Dircksen 2009; Kahsai et al. 2010; Lee et al. 2008)*. I also examined *Treh*, involved in trehalose synthesis, a marker of desiccation resistance (Matsuda *et al.* 2015; Thorat *et al.* 2012) insulin receptor *InR* involved in metabolic homeostasis and survival to desiccation (Söderberg *et al.* 2011), and *FASN2* encoding cuticle constituents methyl-

branched cuticular hydrocarbons (mbCHCs) (Gibbs & Pomonis 1995). Less directly associated with *Drosophila* desiccation resistance but expressed in the MTs include hygrosensing Transient Potential Receptors (TRPs) *trp* and *trpl*, (MacPherson *et al.* 2005), and *CG7084*, the most highly expressed gene in the MTs (Wang *et al.* 2004). Finally I examined the expression of *Mtl*, a modulator of the MAPK stress signalling network, shown to be elicited by desiccation stress in human cell lines (Huang & Tunnacliffe 2005). While gene expression under desiccation has not been examined in east Australian populations, previous work mapping allelic polymorphisms associated with desiccation resistance in temperate female *D. melanogaster* revealed variation in candidate genes *Trpl, klu, ITP*, *sNPF*, *InR*, *Treh* and *Mtl* (Telonis-Scott *et al.* 2012; Telonis-Scott *et al.* 2016), and it is of interest to verify if expression variation of these genes is elicited by desiccation stress.

Utilizing a natural study system and candidate gene framework, I addressed the following questions: 1) Do the populations differ in their genetic and plastic responses to desiccation? 2) Can I confirm that the candidate genes are desiccation responsive? 3) Do any transcriptional profiles correspond to phenotypic differences between the populations? 4) Is there a distinct impact of hardening on gene transcription compared to expression in unhardened flies? The latter question is of interest given the well-known effect of non-lethal exposure to heat stress. Previous work has shown that heat hardening significantly improves thermotolerance and results in rapid up-regulation of *Hsp* expression. Flies are effectively 'primed' by up-regulation of key heat responsive genes to defend cellular processes from subsequent hyperthermia (Lindquist 1981, 1986). Temporally, highly elevated *Hsp* priming resulting from heat hardening at time zero prior to subsequent stress can persist for hours and even days (Telonis-Scott *et al.* 2014)

3.3 Materials and Methods

3.3.1 Fly collection and maintenance

Flies were collected from eastern Australia and maintained as described in Clemson *et al.* (2016). Briefly, mass-bred *D. melanogaster* populations from Melbourne ('temperate population', 37.8136° S, 144.9631° E) and Innisfail ('tropical population' 17.5236° S, 146.0292° E) were initiated from 30 field inseminated females. Progeny were mixed into one pool and redistributed into three 250 mL bottles with media each generation to control density. Populations were maintained this way at a census size of approximately 1000 individuals at 25°C under 12:12 light:dark conditions.

3.3.2 Desiccation hardening and mortality assays

Experimental flies (generation F_8 and F_6, temperate and tropical populations respectively) were reared in 250 mL bottles with media and density controlled by restricting parental oviposition to two hours. Progeny were collected at one-two days post-eclosion into mixed sex cohorts and allowed to mate for 48 hours. At three-four days post-eclosion females were sorted into groups of 10 using aspiration without CO_2. Flies from each population were then randomly assigned into two test groups: (i) 'basal' (genetic) resistance or (ii) 'hardened' (plastic) resistance and maintained in their groups in 10-dram vials with 6.5 mL media until the assays.

First, flies allocated to the hardened treatment group were subjected to a 10 hour pre-treatment at <10% relative humidity (RH), which involved transferring groups into empty vials topped with gauze and placing them into a glass desiccator (60 cm x 30 cm x 35 cm; which eliminated dry air flow) containing silica gel at 25^{o}C. After 10 hours of hardening the flies were removed and allowed to recover on media for 10 hours at ambient RH at 25^{o}C (Fig. 3.1). Flies allocated to the basal treatment group were maintained in their vial, on media and ambient RH at 25^{o}C, throughout this time. Next, the desiccation resistance at <10% RH of both treatment groups was measured using a desiccation mortality assay. Briefly, five-six day old individual females were transferred into 5 mL glass vials covered by gauze, and placed into the desiccator containing silica gel at 25^{o}C. The time taken for each fly to die was recorded to the nearest hour. Approximately 30 flies from each treatment/population were assessed for a total of 120 flies.

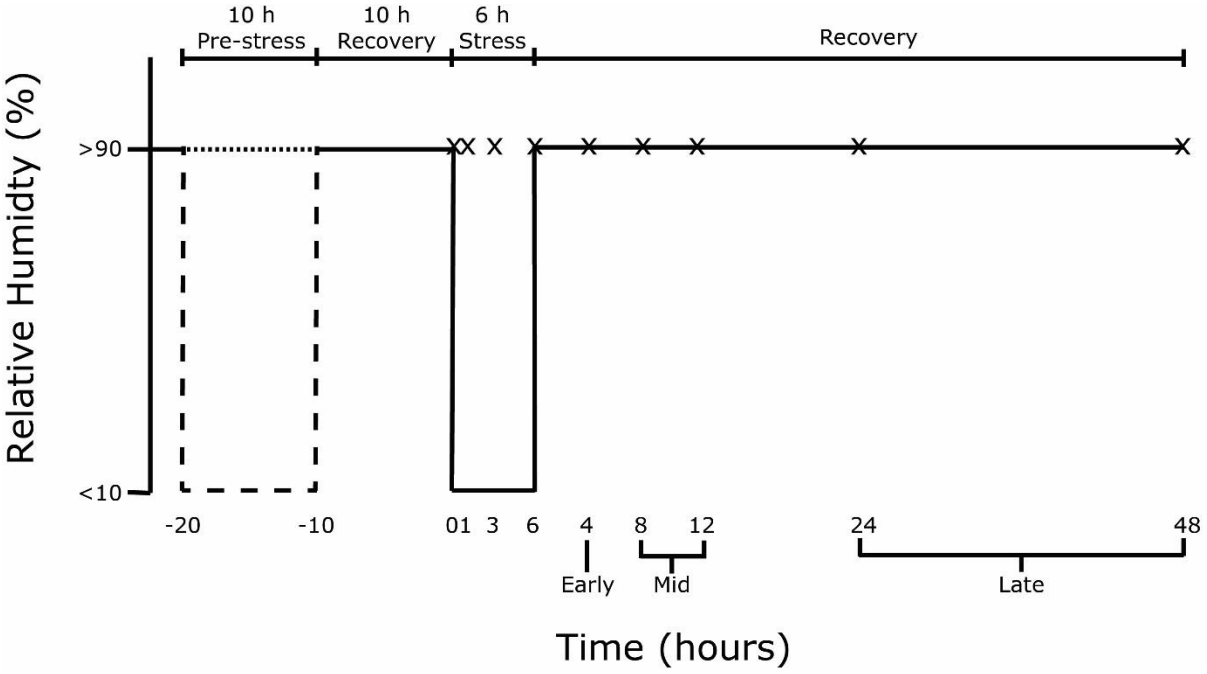


Figure 3.1: Sampling schematic for two desiccation stress treatment profiles. At pre-stress, flies either underwent hardening (dashed line) at <10% RH or remained at >90% RH (basal, dotted line). The first time-point (time zero) represents 20 h following the commencement of hardening (10 hours stress, 10 hours recovery) or constant RH (basal flies). Flies sampled during stress were exposed to <10% RH for 1, 3 or 6 h. Recovery flies were exposed to <10% RH for 6 hours before returning to >90% RH for 4, 8, 12, 24 or 48 h. For analysis, the five 'recovery' time-points were combined to form the early, mid and late recovery time-points. Crosses represent time-points where samples were snap-frozen for gene expression analysis. Flies were maintained and assayed at 25^{o}C.

3.3.3 Gene expression of candidate genes during desiccation and recovery

Primer sequences were designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye *et al.* 2012, Table 3.1).

3.3.3.1 Time-series sampling

While population level desiccation resistance was assessed using a desiccation mortality assay as described above, I aimed to assess the temporal expression dynamics of the candidate genes *prior* to death to avoid signatures of apoptosis. To best determine the sampling time-points during desiccation stress, flies were incrementally subjected to <10% RH (3, 5, 6, 7, 8, 9, 10 and 11 hours stress exposure) at 25^{o}C in 10 groups of 10 females from each population, and assessed for mortality after 48 hours recovery at ambient RH. Mortality began to occur at seven hours of

stress exposure therefore the duration of stress for the transcript analysis (described below) was limited to six hours (data not shown).

Table 3.1: Primer sequences for real-time PCR

Gene	Forward primer	Reverse primer
RpL11	CGATCCCTCCATCGGTATCT	AACCACTTCATGGCATCCTC
Gapdh2	CGTTCATGCCACCACCGCTA	CCACGTCCATCACGCCACAA
CycK	CCCAAAAAGAAGCGCTCCAG	AGCTTGCCGGATTTTGGACT
trpl	GAGCCCTACAGTTGGCAAAA	CCAGCATCAGAGGCGTGATA
trp	TGCCAGGTGTCAAGAAGATCC	TTCATGGGATCCGTGCAGTT
Capa	CGAATTCAGTACAGCTGAGACG	GAAGGCATAGAGCCCCATGTT
CapaR	TCAGAGGCATGCACCTATGTC	TGGCAAATGGCCAGGAATCT
klu	CAAAGAGTCCCAAAAGTCTCGC	TGGCCACAAGATATCCAGCC
ITP	ACCGATTATGCAAGAAAGACTGC	TCCTCTTCGGGTATTAACAGCA
sNPF	GTAAACCAATGCGCTTGCGA	CCTCGGGAGTATGCTTTTGC
Mtl	ATGTGCCCACAGTCTTCGAC	AGTCCCAGCGAGACCTGTAT
InR	AAACCGCGGATCATGTGGAA	CATAGCGGAGTTTGCTCCCA
FASN2	ACCAGGTGCTACTGCAACTC	CGTGTAACCAGCCAGAGTGT
Treh	CCCTCGAGGACTTTAATGCCA	AGTACTTATCGACAAACTGCTTGAG
CG7084	ACAAATGGGCGACTCCTACG	GGTGCACATGATGGACCCAA

For the time-series sampling, flies were reared in controlled densities by placing groups of 50-70 eggs into 10-dram vials containing 6.5 mL of media to develop at 25°C under 12:12 light:dark conditions. Once eclosed, experimental flies were maintained as for the phenotyping assays described above, with flies randomly assigned into 'basal' and 'hardened' groups; hardened flies were subjected to a pre-treatment regime of 6 hours exposure. Groups of 20 female flies were then randomly allocated into time-point groups (for a total of 60 flies per population/treatment/time-point) in the following time-series: 'pre-stress' (time-zero, >90% RH, media); 'stress' at 1, 3 and 6 hours (<10% RH, no media); 'recovery' from 6 hours exposure (<10% RH, no media) at 4, 8, 12, 24, 48 hours post stress (>90% RH, media; Fig. 3.1). Flies in the 'pre-stress' group were placed into 1.7 mL Eppendorf tubes and snap frozen in liquid N_2 immediately at the start of the stress exposure. The 'stress' groups were placed into

empty gauze-covered 15 mL Bunzel cryotubes, sampled immediately at the appropriate time and snap frozen in liquid N_2. The 'recovery' groups were placed into empty gauze-covered 10 dram vials and stressed for six hours, removed and placed on food for the appropriate recovery duration, then transferred to 1.7 mL Eppendorf tubes and snap frozen in liquid N_2. All assays were conducted at 25°C.

3.3.3.2 Quantification of transcript abundance

Total RNA was extracted using a combination of TRIzol® Reagent coupled with the Zymo Direct-zolTM RNA kit (Zymo Research Corporation, Orange, CA), following the manufacturer's instructions. This resulted in 50µL of purified RNA per sample, which was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed with 1% agarose gel electrophoresis. Complimentary DNA (cDNA) was synthesized from 1µg of RNA using the Roche Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN), following the manufacturer's instructions. The final volume was then diluted 1:9 with Diethylpyrocarbonate (DEPC) water before quantitative real-time PCR (qRT-PCR).

PCR was performed using a Roche Lightcycler® 480 and SYBR Green chemistry in 384-well plates assembled using an Eppendorf epMotion® 5075 automated pipetting system. Samples were quantified in duplicate, for a total of three biological replicates per population/treatment/time-point (108 samples in total). As all samples could not fit onto a single 384 well plate, samples were blocked on plates by replicate Transcript abundance was quantified relative to the geometric mean (GM) of three stable 'housekeeping' genes (*RpL11, Gapdh2 and CycK*) using the formula: transcript of interest (TOI) = $2^{(GM - TOI)}$. To confirm that each housekeeper was not affected by desiccation stress and population differences, expression was assayed using one-way analysis of variance (ANOVA; data not shown).

3.3.4 Statistical analysis

3.3.4.1 Phenotypic response to desiccation

Mean desiccation resistance (in hours) and the effect of hardening between the populations was analysed using a two-way mixed model analysis of variance (ANOVA)

with population (temperate or tropical) and treatment (basal or hardened) as fixed factors, individual fly as a random factor and the interaction between population and treatment. Residual diagnostics showed a slight departure from normality (Shapiro-Wilk test p=0.0157) and this was resolved by fitting a mixed model to log transformed data with REPEATED/SUBJECT = individual (population) and the GROUP = treatment statements (PROC MIXED, SAS V9.4) to account for heterogeneity of treatment variances (Telonis-Scott *et al.* 2014). The interaction term was further examined by Tukey's honestly significant difference (HSD) *post hoc* tests.

3.3.4.2 Transcript level response to desiccation

The effect of population (temperate or tropical) and treatment regime (basal or hardened) on the mean raw temporal expression (time-point) of each gene was examined using a three-way fixed-effects ANOVA. Residual diagnostics were performed using Shapiro-Wilk test and Levene's tests and several genes showed departures from normality, therefore the data were log transformed to improve linearity. The effect of treatment was explored using planned contrasts by comparing expression within each population and between treatments at each time-point (e.g. temperate basal *vs.*. temperate hard at x time-point), resulting in a total of 14 contrasts per gene. There was negligible variation between the 8 and 12 hour recovery time-points and the 24 and 48 hour recovery time-points (not shown), and for brevity data were pooled to create mid- (8-12 hours) and late- (24-48 hours) recovery time-points respectively. For consistency, I refer to the 4 hour time-point as 'early-recovery'. *P*-values were corrected for multiple tests using a false discovery rate (FDR) approach (Benjamini & Hochberg 1995).

I also examined differences between pre-stress expression and stress/recovery expression within each population and treatment group using Dunnett's multiple comparison one-way ANOVAs. This resulted in a total of 20 comparisons per gene (5 for each population/treatment group combination). The average $\log_2$ fold-change scaled to time-zero for each time-point from each population/treatment group are shown for illustrative purposes.

3.4 RESULTS

3.4.0.1 Temperate and tropical females differ in their genetic and plastic phenotypic responses to desiccation

Desiccation resistance differed between the populations and treatment groups (Table 3.2). The temperate females had higher basal resistance, surviving on average a further 3.2 hours than tropical females (Fig. 3.2, Tukey's HSD, $p < 0.001$). Hardening improved desiccation resistance by 1.2 hours for temperate females and 2.8 hours for tropical females (7% and 19.5% increase, temperate and tropical populations respectively, Fig. 3.2; within population comparisons: Tukey's HSD, $p < 0.001$).

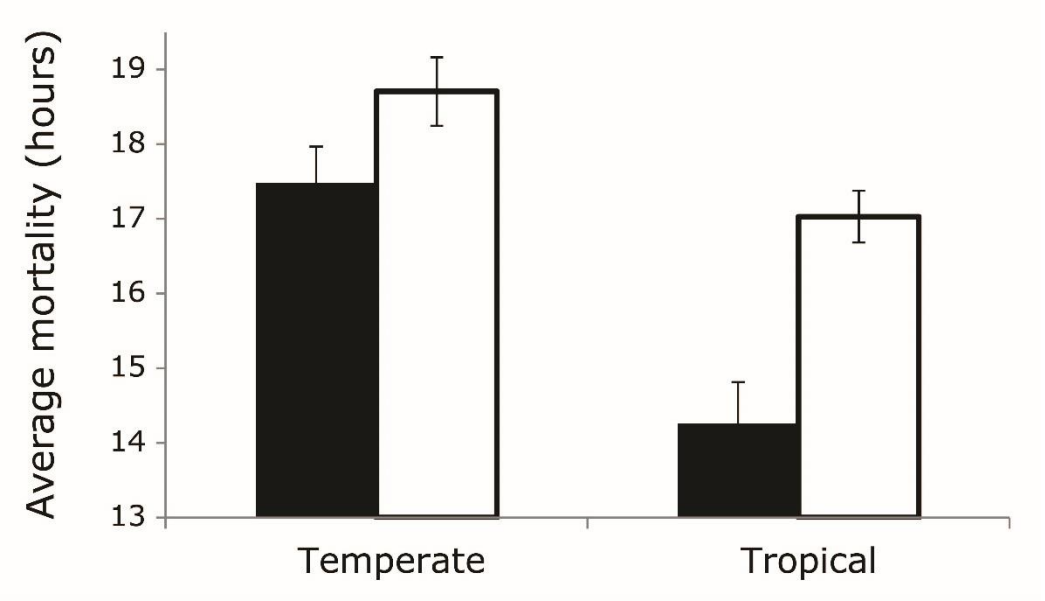


Figure 3.2: Average mortality resulting from desiccation of individual female *D. melanogaster* from two populations from eastern Australia. Black bars represent unhardened treatment groups ('basal' resistance) and white bars represent hardened treatment groups. Average desiccation resistance was significantly different within treatments and between the populations (Tukey's HSD $p < 0.001$) except for the 'unhardened' temperate group compared to the 'hardened' tropical group where resistance was similar (Tukey's HSD $p = 0.5452$). Error bars represent standard error of the mean.

The population-by-treatment interaction term was also significant (Table 3.2), largely driven by the differences in hardening responses, although the temperate population still withstood desiccation significantly longer than the tropical population following hardening (Fig. 3.2, Tukey's HSD, $p < 0.001$). Despite the tropical females' larger

plastic response, their hardened desiccation resistance was comparable to the mean resistance of the unhardened temperate females (Fig. 3.2, Tukey's HSD, *P* =0.55).

Table 3.2: Two-way mixed model analyses of variance (ANOVA) with fixed effects of population and treatment (basal or hardened), and the interaction term for mean desiccation resistance. Significant terms are bolded.

Source	df	F	*P*
Population	1	21.1	**4.13E-07**
Treatment	1	28.4	**1.01E-05**
Population × Treatment	1	4.82	**0.0298**
Error	131		

3.4.1 Transcript level responses to desiccation

3.4.1.1 Desiccation elicits expression of the candidate genes

Three-way ANOVA with the fixed effects of population, treatment and time-point revealed that all 12 genes exhibited significant temporal variation in transcript abundance following desiccation exposure, with a significant effect of time for all transcripts (Table 3.3; Fig. 3.3A-L).

3.4.1.2 Low levels of gene expression population differentiation

Three-way ANOVA revealed that the population term was significant for only one gene encoding cuticle mbCHCs, *FASN2* where overall expression was higher in the tropical population (Table 3.3; Fig. 3.3J). Planned contrasts between the populations for pre-stress expression levels indicated genetic (basal) differences prior to stress (time-zero, temperate *vs.* tropical FDR <0.01) although this difference disappeared following hardening (Fig. S3.1; Table S3.1). Compared to unhardened temperate females, unhardened tropical females had significantly upregulated *FASN2* expression at 6 hours stress, and remained significantly elevated into recovery ('basal' temperate *vs.* tropical 6 hours stress, early, mid and late recovery FDR <0.01, Fig. S3.1; Table S3.1). This was also the case in hardened flies where hardening elicited expression of *FASN2* in both populations but to a greater extent in the tropical females; at 1 and 6 hours desiccation stress ('hardened' temperate *vs.* tropical FDR < 0.001 and <0.05 respectively, Fig.S3.1; Table S3.1) and during mid- and late-recovery (FDR <0.01, Fig. S3.1; Table S3.1).

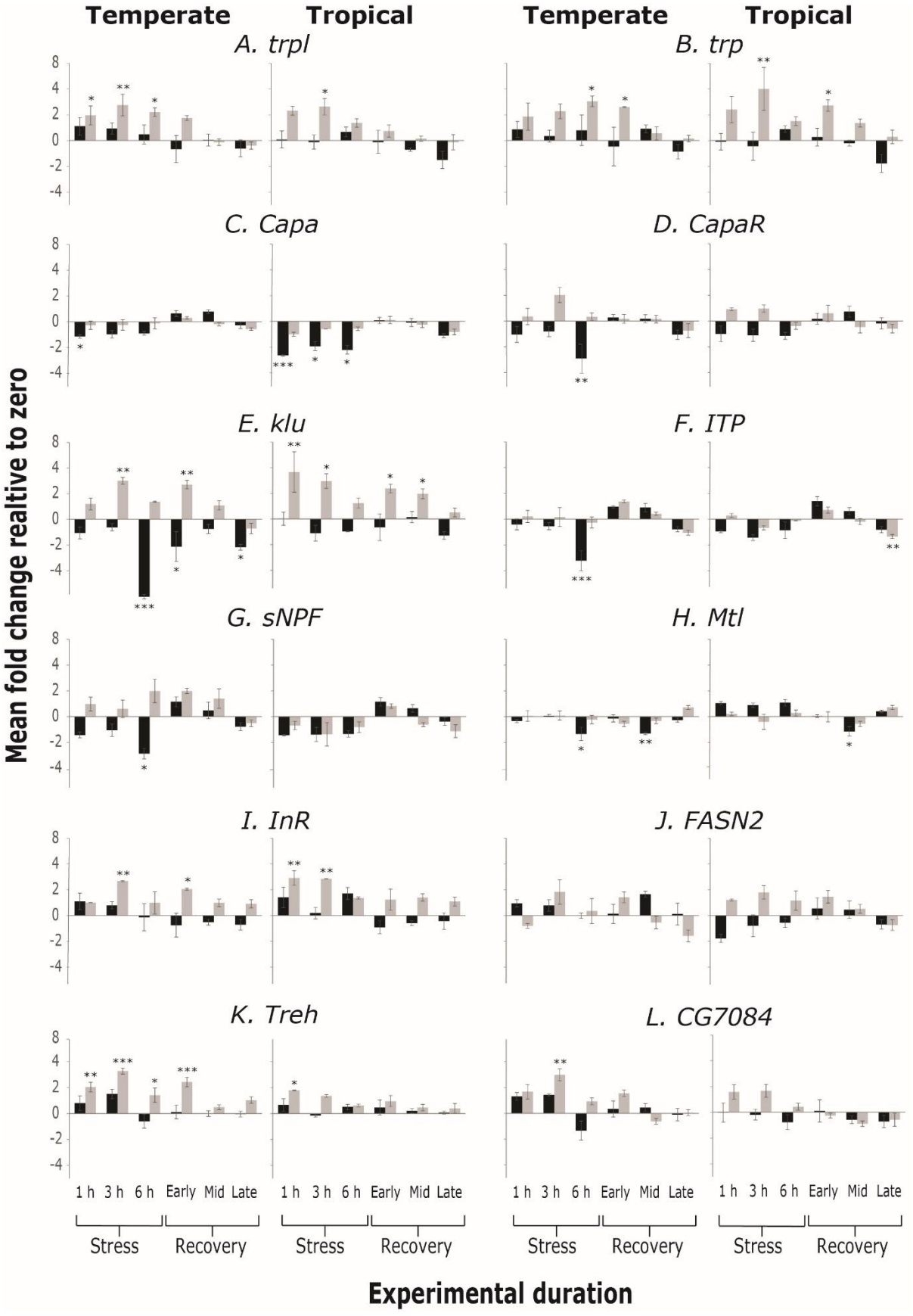

Temperate
Tropical
Temperate
Tropical
A. trpl
B. trp
C. Capa
D. CapaR
E. klu
F. ITP
G. sNPF
H. Mtl
I. InR
J. FASN2
K. Treh
L. CG7084
Mean fold change realtive to zero
1 h 3 h 6 h Early Mid Late
Stress
Recovery
Experimental duration

Figure 3.3: Geographic and temporal variation in transcript abundance of 12 desiccation resistance candidate genes (*trpl, trp, Capa, CapaR, klu, ITP, sNPF, Mtl, InR, FASN2, Treh, CG7084*; A-L) exposed to two stress regimes. Expression is relative to pre-stress expression. Black lines = basal expression (no hardening pre-treatment), grey lines = expression following 10 hours exposure <10% RH and 10 hour recovery at >90% RH. Error bars represent ± SE of the mean. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 for Dunnett's multiple comparison one-way ANOVAs.

One gene, *ITP* encoding an antidiuretic peptide showed a significant population-by-time-point interaction (Fig. 3.3F Table 3.3), largely driven by significant down-regulation at 6 hours stress in temperate females compared to tropical females (6 h stress temperate *vs.* tropical FDR <0.01 Fig. S3.1; Table S3.1). There were slight differences during stress between the populations without hardening; 6 hours stress elicited significantly higher expression of the MAPK kinase *Mtl* in tropical females compared to temperate females, and at 3 hours stress, higher expression of *treh* in temperate females ('basal' temperate *vs.* tropical FDR <0.01 and 0.05 *Mtl* and *treh* respectively, Fig. S3.1; Table S3.1).

3.4.1.3 Hardening elicits a distinct molecular profile in most genes

In the three-way ANOVA, the treatment term was significant for all genes barring *trpl*, *trp* and *InR* (Table 3.3). The effect of treatment varied temporally, where all genes except *trpl* exhibited significant treatment-by-time-point interactions (Table 3.3). For each gene and population, planned contrasts were used to examine whether hardening resulted in gene 'priming', (*i.e.* increased expression) by examining pre-stress expression levels between the basal and hardened treatments prior to desiccation stress. I found no evidence that hardening increased expression levels prior to subsequent stress; rather, expression was lower following hardening for *klu* in both populations and *Mtl* in temperate females (*klu*; basal *vs.* hardened pre-stress temperate and tropical females respectively FDR <0.05, FDR 0.06; *Mtl* FDR <0.05, Fig. S3.1; Table S3.2).

Despite the lack of expression priming following the non-lethal pre-stress treatment, hardening elicited distinctive transcriptional responses compared to untreated flies during desiccation stress, with most genes returning to pre-stress levels upon early-mid recovery (Fig. 3.3A-L) Given the large number of potential comparisons, for brevity

I restricted contrasts between the treatments for each gene and population at each of the six time-points. To provide scaled expression values for the genes, I also characterised the temporal profiles relative to pre-stress for each treatment and population using Dunnett's multiple comparison one-way ANOVAs (Fig. 3.3A-L). The latter analyses revealed that fold-changes relative to stress were relatively modest and largely similar in magnitude between the populations.

However, I did find that the genes tended to display one of two expression profiles: 1) an increase in expression following the hardening treatment with expression in unhardened flies remaining relatively consistent (*trpl, trp, klu*, *InR*, *FASN2*, *Treh* and *CG7084*; Fig. 3.3A-B, E & I-L; Table S3.3) or 2) a decrease in expression in unhardened flies with expression in hardened flies remaining relatively consistent (*Capa*, *CapaR*, *ITP, sNPF* and *Mtl*; Fig. 3.3C-D & F-H; Table S3.3).

Table 3.3: Three-way fixed-effects general linear model ANOVA for the effects of population, treatment (basal or hardened) and time-point on transcript abundance of 12 desiccation resistance candidate genes during and in recovery from <10% RH stress. Significant terms are bolded.

Transcript	Source of variation	*df*	*SS*	*F*	*p*	*Adj. p*
trpl	Population	1	0	0.0006	0.98098	0.98098
	Treatment	1	1.1	4.1735	0.04462	0.05354
	Time	6	15.6	9.5472	2.63E-06	**1.85E-07**
	Population × Treat	1	0	0.1569	0.69314	0.95113
	Population × Time	6	0.1	0.0763	0.99820	0.99820
	Treatment × Time	6	2.9	1.7996	0.11082	0.11082
	Population × Treatment × Time	6	1.6	1.0028	0.43012	0.57895
	Error	74	20.1			
trp	Population	1	1.9	5.2364	0.02498	0.14988
	Treatment	1	0.9	2.3943	0.12604	0.13750
	Time	6	15.7	7.3359	3.56E-06	**4.75E-06**
	Population × Treat	1	0.1	0.3007	0.58509	0.95113
	Population × Time	6	0.8	0.3724	0.89428	0.99820
	Treatment × Time	6	5.7	2.6825	0.02067	**0.02407**
	Population × Treatment × Time	6	2.5	1.1503	0.34226	0.57895
	Error	74	26.4			
Capa	Population	1	0.2	2.4333	0.12310	0.37012
	Treatment	1	3.4	47.9239	1.37E-09	**1.64E-08**
	Time	6	7	16.1837	8.44E-12	**5.06E-11**
	Population × Treat	1	0	0.0012	0.97280	0.97280
	Population × Time	6	0.5	1.2618	0.28550	0.65477
	Treatment × Time	6	2.9	6.6049	0.00001	**0.00008**

	Population × Treatment × Time	6	0.1	0.2539	0.95620	0.95620
	Error	74	5.3			
CapaR	Population	1	0.24	2.3845	0.12681	0.97381
	Treatment	1	0.64	6.4186	0.01341	**0.00001**
	Time	6	5.81	9.7513	6.72E-08	**0.00432**
	Population × Treat	1	0.01	0.1127	0.73803	0.95113
	Population × Time	6	1.83	3.0719	0.00969	0.99820
	Treatment × Time	6	1.58	2.649	0.02206	**0.00083**
	Population × Treatment × Time	6	0.72	1.2096	0.31104	0.65095
	Error	74	7.35			
klu	Population	1	0.1	0.6659	0.41719	0.62579
	Treatment	1	2.8	13.0633	0.00055	**0.00083**
	Time	6	14	10.953	1.23E-08	**3.69E-08**
	Population × Treat	1	0	0.0516	0.82090	0.95113
	Population × Time	6	2.5	1.9675	0.08156	0.24468
	Treatment × Time	6	10.7	8.3867	0.00000	**0.00001**
	Population × Treatment × Time	6	3.1	2.4546	0.03240	0.38880
	Error	72	15.3			
ITP	Population	1	0.2	1.7897	0.18506	0.37012
	Treatment	1	3.2	30.4429	4.83E-07	**1.16E-06**
	Time	6	13.9	21.9403	1.18E-14	**1.42E-13**
	Population × Treat	1	0	0.0262	0.87187	0.95113
	Population × Time	6	1.8	2.8068	0.01624	**0.06496**
	Treatment × Time	6	3.9	6.252	0.00002	**0.00010**
	Population × Treatment × Time	6	0.9	1.403	0.22490	0.53976
	Error	74	7.8			
sNPF	Population	1	0.3	1.2406	0.26897	0.46108
	Treatment	1	7.1	30.5755	4.60E-07	**1.16E-06**
	Time	6	12.8	9.2297	1.53E-07	**2.30E-07**
	Population × Treat	1	0.3	1.3384	0.25104	0.95113
	Population × Time	6	1	0.707	0.64490	0.96735
	Treatment × Time	6	5.7	4.1112	0.00129	**0.00310**
	Population × Treatment × Time	6	2	1.4422	0.21018	0.53976
	Error	74	17.1			
Mtl	Population	1	0.13	1.8896	0.17339	0.37012
	Treatment	1	2.14	32.3917	2.39E-07	**9.56E-07**
	Time	6	4.99	12.5753	1.01E-09	**4.04E-09**
	Population × Treat	1	0.02	0.2483	0.61977	0.95113
	Population × Time	6	1.27	3.1882	0.00773	0.05816
	Treatment × Time	6	1.47	3.7054	0.00283	**0.00485**
	Population × Treatment × Time	6	0.68	1.7135	0.12971	0.53976
	Error	74	4.9			
InR	Population	1	0	0.0931	0.76112	0.97381
	Treatment	1	0.1	0.6852	0.41053	0.41053
	Time	6	7.8	6.1784	0.00003	**0.00003**
	Population × Treat	1	0.2	1.0024	0.32008	0.95113
	Population × Time	6	1.5	1.1786	0.32738	0.65477
	Treatment × Time	6	3.9	3.0981	0.00935	**0.01247**

	Population × Treatment × Time	6	1	0.7592	0.60426	0.65919
	Error	72	15.1			
FASN2	Population	1	31.6	98.9623	3.18E-15	**3.82E-14**
	Treatment	1	8.4	26.2219	2.39E-06	**4.78E-06**
	Time	6	8.2	4.266	0.00097	**0.00106**
	Population × Treat	1	0.1	0.179	0.67345	0.95113
	Population × Time	6	0.5	0.2528	0.95666	0.99820
	Treatment × Time	6	6.5	3.3845	0.00532	**0.00798**
	Population × Treatment × Time	6	3	1.5584	0.17163	0.53976
	Error	73	23.3			
Treh	Population	1	0.24	2.3845	0.12681	0.37012
	Treatment	1	0.64	6.4186	0.01341	**0.01788**
	Time	6	5.81	9.7513	6.72E-08	**1.61E-07**
	Population × Treat	1	0.01	0.1127	0.73803	0.95113
	Population × Time	6	1.83	3.0719	0.00969	0.05816
	Treatment × Time	6	1.58	2.649	0.02206	**0.02407**
	Population × Treatment × Time	6	0.72	1.2096	0.31104	0.57895
	Error	74	7.35			
CG7084	Population	1	0.01	0.0282	0.86699	0.97381
	Treatment	1	7.66	40.7143	1.37E-08	**8.22E-08**
	Time	6	10.53	9.3252	1.32E-07	**2.26E-07**
	Population × Treat	1	0.05	0.2694	0.60525	0.95113
	Population × Time	6	1.12	0.996	0.43449	0.74484
	Treatment × Time	6	4.37	3.8674	0.00207	**0.00413**
	Population × Treatment × Time	6	1.13	0.9964	0.43421	0.57895
	Error	74	13.93			

3.5 DISCUSSION

Despite divergence in desiccation phenotypes between the populations, this could not be discerned at the gene transcript level in key candidate genes linked either directly or indirectly to desiccation resistance. There is evidence that *Drosophila* desiccation resistance is a highly polygenic trait (Foley & Telonis-Scott 2011; Telonis-Scott *et al.* 2012; Telonis-Scott *et al.* 2016) where hundreds of loci of small effect likely contribute to desiccation defence and recovery. While I examined genes from single gene studies reporting large effects on desiccation resistance in laboratory strains such as *Capa (Terhzaz et al. 2014; Terhzaz et al. 2015)*, I was unable to conclusively link expression variation in the candidate genes to natural population level phenotypic divergence. While the genes were desiccation responsive both with and without hardening, my data speaks of the complexity of this trait in wild genetically diverse *Drosophila*. Further research is required to bridge the gap between phenotypic divergence for desiccation stress and the underlying molecular mechanisms utilising combinations of genome-wide methodologies such as full transcriptome sequencing, GWAS, QTL mapping etc. (Telonis-Scott *et al.* 2016)

Interestingly, the expression profiles differed between unhardened and hardened flies, providing the first evidence that desiccation hardening impacts downstream transcriptional patterns. Compared to the well-characterised and ubiquitous heat shock response (for review see Lindquist 1986), I found that the molecular response to desiccation stress with and without hardening is somewhat different. For example, in Australian populations from similar geographies studied here, heat shock proteins (Hsps) are upregulated in the order of 50 to >1000 fold following heat shock (Telonis-Scott *et al.* 2014; Telonis-Scott *et al.* 2013) however expression responses of this magnitude were not observed for the candidates in the present study. I observed only modest fold-changes (not exceeding 8 fold) in the candidate desiccation genes examined here. This is also consistent with work by Sinclair *et al.* (2007) who found basal up-regulation of five (different) candidate genes was of a lower magnitude compared to heat stress. However interpretation of these results may be complicated by the polygenetic nature of the trait, and I cannot preclude the impact of tissue specificity of several of the candidates examined here. For example, *CG7084* is expressed in renal tissues (Wang *et al.* 2004), *FASN2* in oenocytes (Chung *et al.*

2014), *trpl* and *trp* in the head (Chintapalli *et al.* 2007). More research between divergent populations at the tissue level may reveal stronger differential signals and perhaps correspond more robustly to phenotypic divergence among wild flies. Nonetheless, while I used whole flies in my study, I identified distinct molecular profiles between hardened and unhardened flies providing insight into genetic and plastic molecular responses to desiccation stress.

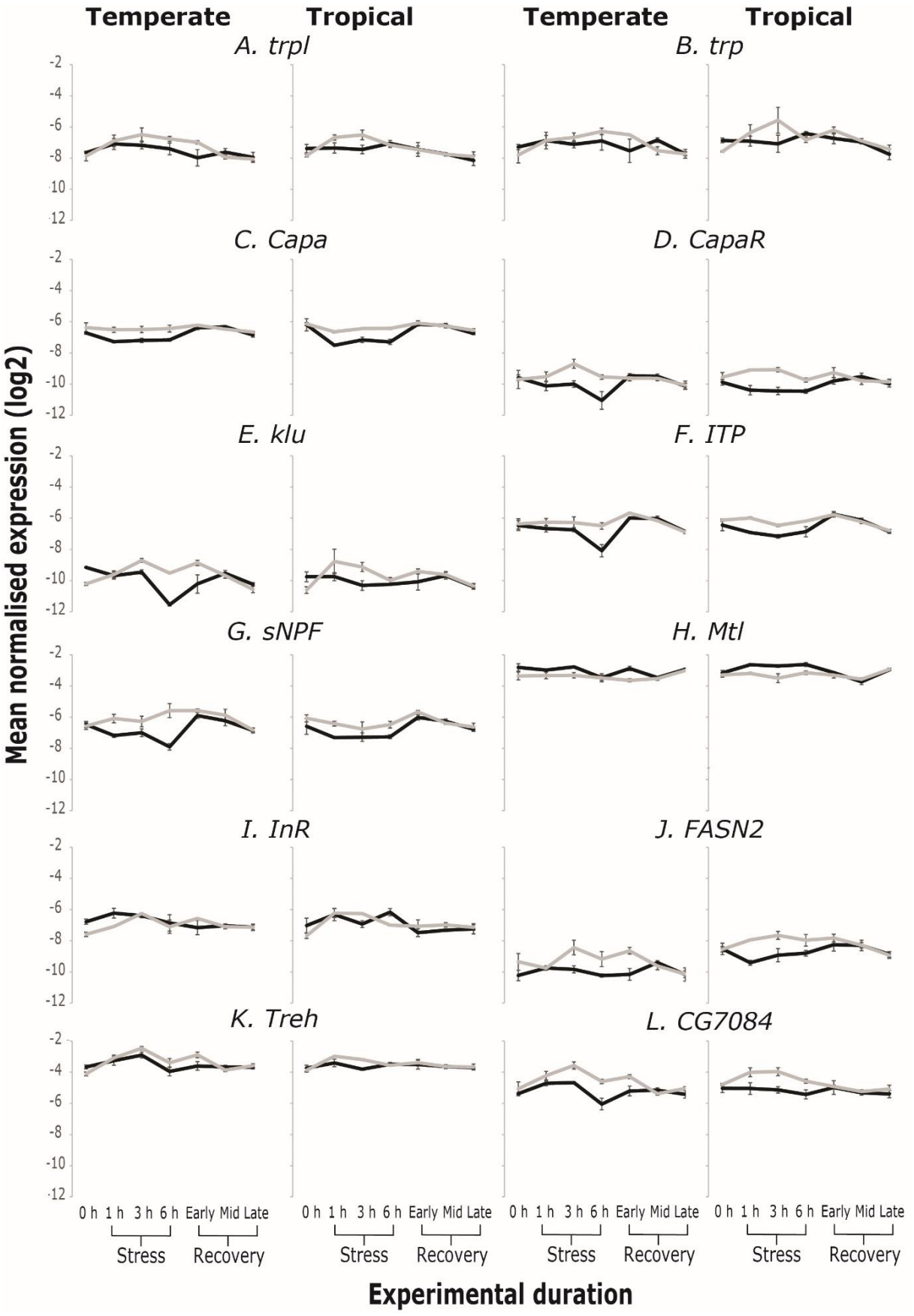
Previous work has shown that non-lethal thermal exposure prior to acute heat stress (heat hardening) significantly improves thermotolerance and results in rapid up-regulation of *Hsp* expression following hardening that is discernible prior to subsequent heat exposure, where flies are effectively 'primed' to defend cellular processes from subsequent hyperthermia (Lindquist 1981, 1986). Temporally, highly elevated *Hsp* priming resulting from heat hardening at time zero prior to subsequent stress can persist for hours and even days (Telonis-Scott *et al.* 2014). Apart from *klu* (both populations)*, Mtl*, *InR* and *FASN2* (temperate population only) where pre-stress expression levels differed between desiccation hardened and unhardened flies, I did not observe this phenomenon. Essentially, at the onset of acute desiccation stress (time-zero) transcript levels were similar between the unhardened and hardened flies unlike heat shock priming. However, desiccation hardening did impact expression patterns during the subsequent acute stress exposures, suggesting that prior exposures to low humidity may prime expression of key desiccation genes. The mechanisms underpinning this response however present an intriguing area of further research. Furthermore, unlike thermal stress, gene expression in response to desiccation stress returned to pre-stress levels rapidly during recovery. Rather, the majority of changes in transcript abundance occurred during desiccation stress itself with little change during recovery from the stress. While still complex, the heat shock response is driven by genes of large effects, while desiccation resistance is more complex. Hardening results in both phenotypic and molecular priming for both stresses, but my data proposes quite different mechanisms underpinning transcriptional priming. While I did not examine *Hsps* as there is little evidence to suggest that they are involved in the response to desiccation to date, Sinclair *et al.* (2007) examined *Hsps* in unhardened *D.* melanogaster and found no significant effect of desiccation on *Hsp* expression.

Finally, several genes with known roles in fluid transportation (*trpl* and *trp*) and diuresis (*Capa*) appear to have the opposite expression profile to what might be expected based on previous work. Specifically, up-regulation of these genes might be expected to lead to an increase in fluid transport, increase in water loss rate and reduced desiccation resistance (MacPherson *et al.* 2005; Terhzaz *et al.* 2015). However, I found these genes to be most highly expressed in the more desiccation resistant hardened flies, and basal expression of *Capa* was actually down-regulated during desiccation stress. However, Terhzaz *et al.* (2014) suggest that increased diuretic activity does not necessarily equate to increased water loss. Instead, diuretic activity during desiccation could be facilitating downstream counter-stress measures including re-uptake of primary urine (Terhzaz *et al.* 2014). Were this to lead to reduced water loss, increases in the expression of *trpl*, *trp* and *Capa* expression could then confer increased desiccation resistance, consistent with the results of the current study.

3.5.1 CONCLUSION

I have found evidence for desiccation-induced plasticity at both the phenotypic and molecular level in *D. melanogaster*. I found that there is significant geographic phenotypic divergence in both basal and plastic desiccation resistance. While temperate populations had higher basal resistance, the tropical population was able to mount a larger plastic response. In contrast, I did not see this geographic divergence at the molecular level; the results suggest that the complex physiology of water balance makes linking gene expression responses to basal and hardened desiccation stress resistance difficult. Nevertheless all genes examined responded to desiccation stress, and ten of the twelve genes were also shown to exhibit strong plastic responses to desiccation stress that differed in flies not exposed to a pre-stress suggesting some level of expression priming that is perhaps unique to desiccation stress. Given the fact that previous genomic studies (Kang *et al.* 2016; Telonis-Scott *et al.* 2016) have identified hundreds of genes that respond to desiccation stress, future research incorporating the whole transcriptome, with tissue-specific transcript analyses, will go a long way towards understanding the molecular underpinnings of innate desiccation resistance among different population and species and the potential for hardening to mitigate exposure to low humidity conditions.

3.6 Supplementary Information



Supplementary Figure 3.1: Geographic and temporal variation in transcript abundance of 12 desiccation resistance candidate genes exposed to two stress regimes. Expression is normalised to the geometric mean of three stable genes: *CycK*, *Gapdh2* and *RpL11* and log2 transformed. Black lines = basal expression (no hardening pre-treatment), grey lines = expression following 10 hours exposure <10% RH and 10 hour recovery at >90% RH. Error bars represent ± SE of the mean.

Supplementary Table 3.1: Planned contrast of the population-by-time-point interaction in 12 candidate genes for desiccation tolerance. An FDR of <0.05 was used to determine statistical significance. Significant terms are bolded.

Contrast	Gene	Treatment	Time	*p*	Adj. *p*
Temperate vs. Tropical	*trpl*	Basal	Pre-stress	0.545	0.818
			1 h stress	0.553	0.818
			3 h stress	0.535	0.818
			6 h stress	0.46	0.818
			Early recovery	0.212	0.818
			Mid-recovery	0.701	0.818
			Late recovery	0.55	0.818
		Hardened	Pre-stress	0.933	0.964
			1 h stress	0.647	0.818
			3 h stress	0.964	0.964
			6 h stress	0.374	0.818
			Early recovery	0.272	0.818
			Mid-recovery	0.584	0.818
			Late recovery	0.596	0.818
Temperate vs. Tropical	*trp*	Basal	Pre-stress	0.382	0.69
			1 h stress	0.9244	0.941
			3 h stress	0.9381	0.941
			6 h stress	0.3874	0.69
			Early recovery	0.1067	0.498
			Mid-recovery	0.7108	0.905
			Late recovery	0.9407	0.941
		Hardened	Pre-stress	0.6399	0.896
			1 h stress	0.3613	0.69
			3 h stress	0.07	0.498
			6 h stress	0.276	0.69
			Early recovery	0.5596	0.87
			Mid-recovery	0.0862	0.498
			Late recovery	0.394	0.69
Temperate vs. Tropical	*Capa*	Basal	Pre-stress	**0.0204**	0.285
			1 h stress	0.2954	0.783
			3 h stress	0.8639	0.93
			6 h stress	0.5817	0.783
			Early recovery	0.2866	0.783
			Mid-recovery	0.6153	0.783
			Late recovery	0.5365	0.783
		Hardened	Pre-stress	0.288	0.783
			1 h stress	0.6054	0.783
			3 h stress	0.8205	0.93
			6 h stress	0.9541	0.954
			Early recovery	0.5233	0.783
			Mid-recovery	0.224	0.783
			Late recovery	0.4753	0.783

Temperate vs. Tropical	*CapaR*	Basal	Pre-stress	0.468	0.725
			1 h stress	0.502	0.725
			3 h stress	0.273	0.725
			6 h stress	0.192	0.725
			Early recovery	0.392	0.725
			Mid-recovery	0.955	0.955
			Late recovery	0.621	0.725
		Hardened	Pre-stress	0.713	0.768
			1 h stress	0.341	0.725
			3 h stress	0.448	0.725
			6 h stress	0.617	0.725
			Early recovery	0.367	0.725
			Mid-recovery	0.601	0.725
			Late recovery	0.433	0.725
Temperate vs. Tropical	*klu*	Basal	Pre-stress	0.11213	0.3924
			1 h stress	0.87084	0.8708
			3 h stress	**0.02616**	0.1831
			6 h stress	**0.00617**	0.0864
			Early recovery	0.72046	0.8405
			Mid-recovery	0.55799	0.7256
			Late recovery	0.5701	0.7256
		Hardened	Pre-stress	0.30096	0.6019
			1 h stress	**0.04954**	0.2312
			3 h stress	0.36174	0.633
			6 h stress	0.21721	0.5068
			Early recovery	0.14811	0.4147
			Mid-recovery	0.85487	0.8708
			Late recovery	0.41502	0.6456
Temperate vs. Tropical	*ITP*	Basal	Pre-stress	0.916201	0.97114
			1 h stress	0.355679	0.75175
			3 h stress	0.120125	0.75175
			6 h stress	**0.000106**	**0.00148**
			Early recovery	0.360365	0.75175
			Mid-recovery	0.556027	0.85844
			Late recovery	0.971139	0.97114
		Hardened	Pre-stress	0.375875	0.75175
			1 h stress	0.36599	0.75175
			3 h stress	0.574909	0.85844
			6 h stress	0.244073	0.75175
			Early recovery	0.714568	0.85844
			Mid-recovery	0.735806	0.85844
			Late recovery	0.617839	0.85844
Temperate vs. Tropical	*sNPF*	Basal	Pre-stress	0.7612	0.883
			1 h stress	0.7484	0.883
			3 h stress	0.4698	0.868
			6 h stress	0.1458	0.68
			Early recovery	0.7582	0.883
			Mid-recovery	0.8834	0.883

			Late recovery	0.7872	0.883
		Hardened	Pre-stress	0.2038	0.713
			1 h stress	0.4544	0.868
			3 h stress	0.318	0.868
			6 h stress	**0.0243**	0.34
			Early recovery	0.8311	0.883
			Mid-recovery	0.0864	0.605
			Late recovery	0.496	0.868
Temperate vs. Tropical	*Mtl*	Basal	Pre-stress	0.095816	0.29329
			1 h stress	0.110706	0.29329
			3 h stress	0.80572	0.8677
			6 h stress	**0.000509**	**0.00712**
			Early recovery	0.209679	0.41936
			Mid-recovery	0.072494	0.29329
			Late recovery	0.893357	0.89336
		Hardened	Pre-stress	0.756138	0.8677
			1 h stress	0.555342	0.86387
			3 h stress	0.484854	0.8485
			6 h stress	0.125694	0.29329
			Early recovery	0.109648	0.29329
			Mid-recovery	0.7671	0.8677
			Late recovery	0.644607	0.8677
Temperate vs. Tropical	*InR*	Basal	Pre-stress	0.52	0.945
			1 h stress	0.81	0.945
			3 h stress	0.151	0.654
			6 h stress	0.139	0.654
			Early recovery	0.384	0.896
			Mid-recovery	0.291	0.815
			Late recovery	0.714	0.945
		Hardened	Pre-stress	0.808	0.945
			1 h stress	0.064	0.654
			3 h stress	0.993	0.993
			6 h stress	0.796	0.945
			Early recovery	0.187	0.654
			Mid-recovery	0.673	0.945
			Late recovery	0.993	0.993
Temperate vs. Tropical	*FASN2*	Basal	Pre-stress	**0.000442**	**0.00145**
			1 h stress	0.462808	0.46281
			3 h stress	0.051996	0.07279
			6 h stress	**0.007196**	**0.01259**
			Early recovery	**0.000098**	**0.00137**
			Mid-recovery	**0.00121**	**0.00242**
			Late recovery	**0.000517**	**0.00145**
		Hardened	Pre-stress	0.087108	0.10163
			1 h stress	**0.000803**	**0.00187**
			3 h stress	0.169485	0.18252
			6 h stress	**0.010945**	**0.01703**
			Early recovery	0.078012	0.09929

			Mid-recovery	**0.00023**	**0.00145**
			Late recovery	**0.000349**	**0.00145**
Temperate vs. Tropical	*Treh*	Basal	Pre-stress	0.763854	0.8912
			1 h stress	0.572084	0.8912
			3 h stress	**0.000718**	**0.0101**
			6 h stress	0.106819	0.3739
			Early recovery	0.715438	0.8912
			Mid-recovery	0.867611	0.8961
			Late recovery	0.896102	0.8961
		Hardened	Pre-stress	0.354186	0.8264
			1 h stress	0.69346	0.8912
			3 h stress	**0.024339**	0.1704
			6 h stress	0.552666	0.8912
			Early recovery	0.056287	0.2627
			Mid-recovery	0.236228	0.6614
			Late recovery	0.66113	0.8912
Temperate vs. Tropical	*CG7084*	Basal	Pre-stress	0.3426	0.781
			1 h stress	0.3496	0.781
			3 h stress	0.1925	0.781
			6 h stress	0.1233	0.781
			Early recovery	0.5377	0.781
			Mid-recovery	0.4821	0.781
			Late recovery	0.9209	0.969
		Hardened	Pre-stress	0.4534	0.781
			1 h stress	0.5888	0.781
			3 h stress	0.3669	0.781
			6 h stress	0.969	0.969
			Early recovery	0.0764	0.781
			Mid-recovery	0.6133	0.781
			Late recovery	0.8763	0.969

Supplementary Table 3.2: Planned contrast of the treatment-by-time-point interaction in 12 candidate genes for desiccation tolerance. An FDR of <0.05 was used to determine statistical significance. Significant terms are bolded.

Contrast	Gene	Population	Time	*P*	Adj. P
Basal vs Hardened	*trpl*	Temperate	Pre-stress	0.6051	0.722
			1 h stress	0.6387	0.722
			3 h stress	0.1525	0.356
			6 h stress	0.1368	0.356
			Early recovery	0.0232	0.162
			Mid-recovery	0.3314	0.58
			Late recovery	0.7224	0.722
		Tropical	Pre-stress	0.3006	0.702
			1 h stress	0.1613	0.565
			3 h stress	0.0546	0.382
			6 h stress	0.8446	0.962
			Early recovery	0.962	0.962
			Mid-recovery	0.9498	0.962
			Late recovery	0.4451	0.779
Basal vs Hardened	*trp*	Temperate	Pre-stress	0.3004	0.526
			1 h stress	0.986	0.999
			3 h stress	0.41	0.574
			6 h stress	0.2141	0.499
			Early recovery	0.0394	0.216
			Mid-recovery	0.0617	0.216
			Late recovery	0.9987	0.999
		Tropical	Pre-stress	0.15058	0.5265
			1 h stress	0.3263	0.5265
			3 h stress	0.00702	**0.0491**
			6 h stress	0.46767	0.5456
			Early recovery	0.29731	0.5265
			Mid-recovery	0.83696	0.837
			Late recovery	0.37605	0.5265
Basal vs Hardened	*Capa*	Temperate	Pre-stress	0.127908	0.22384
			1 h stress	0.000779	**0.00511**
			3 h stress	0.005559	**0.01297**
			6 h stress	0.00146	**0.00511**
			Early recovery	0.451134	0.45113
			Mid-recovery	0.361308	0.42153
			Late recovery	0.235756	0.33006
		Tropical	Pre-stress	0.811698	0.856
			1 h stress	0.000655	**0.00229**
			3 h stress	0.004228	**0.00987**
			6 h stress	0.000647	**0.00229**
			Early recovery	0.745616	0.856
			Mid-recovery	0.856003	0.856
			Late recovery	0.232842	0.40747

Basal vs Hardened	*CapaR*	Temperate	Pre-stress	0.786116	0.86303
			1 h stress	0.148241	0.34589
			3 h stress	0.005023	**0.01758**
			6 h stress	0.000333	**0.00233**
			Early recovery	0.678913	0.86303
			Mid-recovery	0.674885	0.86303
			Late recovery	0.863032	0.86303
		Tropical	Pre-stress	0.41153	0.4801
			1 h stress	0.00535	**0.0187**
			3 h stress	0.00322	**0.0187**
			6 h stress	0.11374	0.2654
			Early recovery	0.18026	0.3155
			Mid-recovery	0.35265	0.4801
			Late recovery	0.6749	0.6749
Basal vs Hardened	*klu*	Temperate	Pre-stress	0.006352	**0.01482**
			1 h stress	0.869929	0.86993
			3 h stress	0.077658	0.1359
			6 h stress	8.29E-06	**5.80E-05**
			Early recovery	0.000595	**0.00208**
			Mid-recovery	0.613339	0.71556
			Late recovery	0.219712	0.3076
		Tropical	Pre-stress	0.02785	0.065
			1 h stress	0.02289	0.065
			3 h stress	0.00628	**0.0439**
			6 h stress	0.55195	0.7727
			Early recovery	0.08072	0.1413
			Mid-recovery	0.80423	0.8641
			Late recovery	0.86411	0.8641
Basal vs Hardened	*ITP*	Temperate	Pre-stress	0.686	0.8
			1 h stress	0.12	0.289
			3 h stress	0.124	0.289
			6 h stress	6.81E-08	**4.77E-07**
			Early recovery	0.236	0.413
			Mid-recovery	0.494	0.692
			Late recovery	0.861	0.861
		Tropical	Pre-stress	0.23718	0.4151
			1 h stress	0.00237	**0.0166**
			3 h stress	0.02167	0.0544
			6 h stress	0.0233	0.0544
			Early recovery	0.92607	0.9261
			Mid-recovery	0.62968	0.8815
			Late recovery	0.78472	0.9155
Basal vs Hardened	*sNPF*	Temperate	Pre-stress	0.79484	0.9104
			1 h stress	0.00664	0.0232
			3 h stress	0.10116	0.236
			6 h stress	8.99E-08	**6.29E-07**
			Early recovery	0.42469	0.5946
			Mid-recovery	0.22868	0.4002

			Late recovery	0.9104	0.9104
		Tropical	Pre-stress	0.1889	0.404
			1 h stress	0.0455	0.279
			3 h stress	0.2307	0.404
			6 h stress	0.0798	0.279
			Early recovery	0.3723	0.521
			Mid-recovery	0.6886	0.689
			Late recovery	0.6263	0.689
Basal vs Hardened	*Mtl*	Temperate	Pre-stress	0.010131	**0.03546**
			1 h stress	0.098443	0.17227
			3 h stress	0.02223	0.05187
			6 h stress	0.988253	0.98825
			Early recovery	0.000516	0.00361
			Mid-recovery	0.682027	0.7957
			Late recovery	0.646308	0.7957
		Tropical	Pre-stress	0.52391	0.61122
			1 h stress	0.02153	0.06184
			3 h stress	0.00138	**0.00964**
			6 h stress	0.0265	0.06184
			Early recovery	0.45729	0.61122
			Mid-recovery	0.28416	0.49728
			Late recovery	0.89157	0.89157
Basal vs Hardened	*InR*	Temperate	Pre-stress	0.0339	0.152
			1 h stress	0.0433	0.152
			3 h stress	0.7567	0.96
			6 h stress	0.5565	0.96
			Early recovery	0.1201	0.28
			Mid-recovery	0.8228	0.96
			Late recovery	0.9962	0.996
		Tropical	Pre-stress	0.0825	0.266
			1 h stress	0.827	0.827
			3 h stress	0.1141	0.266
			6 h stress	0.0498	0.266
			Early recovery	0.2683	0.376
			Mid-recovery	0.2076	0.363
			Late recovery	0.724	0.827
Basal vs Hardened	*FASN2*	Temperate	Pre-stress	0.06223	0.1089
			1 h stress	0.99461	0.9946
			3 h stress	0.00839	**0.0294**
			6 h stress	0.02537	0.0592
			Early recovery	0.00161	**0.0113**
			Mid-recovery	0.52642	0.737
			Late recovery	0.94764	0.9946
		Tropical	Pre-stress	0.95784	0.9772
			1 h stress	0.00599	**0.0419**
			3 h stress	0.01615	0.0565
			6 h stress	0.11134	0.2598
			Early recovery	0.34938	0.6114

			Mid-recovery	0.97716	0.9772
			Late recovery	0.88	0.9772
Basal vs Hardened	*Treh*	Temperate	Pre-stress	0.08847	0.2064
			1 h stress	0.49885	0.582
			3 h stress	0.13208	0.2311
			6 h stress	0.03401	0.119
			Early recovery	0.00707	**0.0495**
			Mid-recovery	0.30692	0.4297
			Late recovery	0.60333	0.6033
		Tropical	Pre-stress	0.6239	0.974
			1 h stress	0.135	0.472
			3 h stress	0.0339	0.237
			6 h stress	0.8156	0.974
			Early recovery	0.643	0.974
			Mid-recovery	0.9951	0.995
			Late recovery	0.8351	0.974
Basal vs Hardened	*CG7084*	Temperate	Pre-stress	0.379338	0.396253
			1 h stress	0.168636	0.23609
			3 h stress	0.007224	**0.025283**
			6 h stress	0.000108	**0.000753**
			Early recovery	0.011361	**0.02651**
			Mid-recovery	0.396253	0.396253
			Late recovery	0.135612	0.23609
		Tropical	Pre-stress	0.49681	0.6955
			1 h stress	0.01043	**0.0365**
			3 h stress	0.00432	**0.0302**
			6 h stress	0.03594	0.0839
			Early recovery	0.85785	0.8578
			Mid-recovery	0.73188	0.8539
			Late recovery	0.23785	0.4162

Supplementary Table 3.3: Dunnett's multiple comparison one-way ANOVAs comparing expression of 12 candidate genes for desiccation tolerance at pre-stress (time-zero) and six experimental time-points. An FDR of <0.05 was used to determine statistical significance. Significant terms are bolded.

Gene	Population	Treatment	Time	*p*	Adj. *p*
trpl	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.309	0.771
			3 h stress	0.390	0.868
			6 h stress	0.659	0.993
			Early recovery	0.536	0.965
			Mid-recovery	0.965	1.000
			Late recovery	0.513	0.955
		Hardened	Pre-stress	-	-
			1 h stress	**0.008**	**0.035**
			3 h stress	**0.001**	**0.007**
			6 h stress	**0.003**	**0.016**
			Early recovery	**0.015**	0.065
			Mid-recovery	0.821	1.000
			Late recovery	0.507	0.955
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.929	1.000
			3 h stress	0.894	1.000
			6 h stress	0.505	0.954
			Early recovery	0.900	1.000
			Mid-recovery	0.360	0.843
			Late recovery	0.069	0.252
		Hardened	Pre-stress	-	-
			1 h stress	**0.012**	**0.054**
			3 h stress	**0.005**	**0.025**
			6 h stress	0.083	0.299
			Early recovery	0.335	0.816
			Mid-recovery	0.829	1.000
			Late recovery	0.837	1.000
trp	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.456	0.923
			3 h stress	0.765	0.999
			6 h stress	0.491	0.945
			Early recovery	0.684	0.996
			Mid-recovery	0.365	0.842
			Late recovery	0.389	0.867
		Hardened	Pre-stress	-	-
			1 h stress	0.052	0.198
			3 h stress	0.036	0.143
			6 h stress	**0.003**	**0.015**

			Early recovery	**0.009**	**0.042**
			Mid-recovery	0.498	0.951
			Late recovery	0.845	1.000
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.924	1.000
			3 h stress	0.652	0.993
			6 h stress	0.420	0.901
			Early recovery	0.783	0.999
			Mid-recovery	0.806	1.000
			Late recovery	0.053	0.200
		Hardened	Pre-stress	-	-
			1 h stress	**0.025**	0.105
			3 h stress	**0.001**	**0.004**
			6 h stress	0.105	0.362
			Early recovery	**0.007**	**0.030**
			Mid-recovery	0.093	0.328
			Late recovery	0.712	0.998
Capa	Temperate	Basal	Pre-stress	-	-
			1 h stress	**0.006**	**0.026**
			3 h stress	**0.016**	0.066
			6 h stress	**0.024**	0.097
			Early recovery	0.094	0.320
			Mid-recovery	**0.022**	0.089
			Late recovery	0.411	0.888
		Hardened	Pre-stress	-	-
			1 h stress	0.541	0.969
			3 h stress	0.625	0.989
			6 h stress	0.787	1.000
			Early recovery	0.497	0.950
			Mid-recovery	0.640	0.991
			Late recovery	0.147	0.469
	Tropical	Basal	Pre-stress	-	-
			1 h stress	**0.000**	**0.001**
			3 h stress	**0.003**	**0.013**
			6 h stress	**0.002**	**0.011**
			Early recovery	0.894	1.000
			Mid-recovery	0.847	1.000
			Late recovery	**0.040**	0.155
		Hardened	Pre-stress	-	-
			1 h stress	**0.031**	0.124
			3 h stress	0.182	0.554
			6 h stress	0.155	0.491
			Early recovery	0.768	0.999
			Mid-recovery	0.446	0.922
			Late recovery	**0.023**	0.095
CapaR	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.200	0.580
			3 h stress	0.320	0.787

			6 h stress	**0.001**	**0.007**
			Early recovery	0.729	0.998
			Mid-recovery	0.794	1.000
			Late recovery	0.135	0.432
		Hardened	Pre-stress	-	-
			1 h stress	0.711	0.997
			3 h stress	0.067	0.246
			6 h stress	0.729	0.998
			Early recovery	0.862	1.000
			Mid-recovery	0.859	1.000
			Late recovery	0.363	0.846
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.193	0.573
			3 h stress	0.153	0.482
			6 h stress	0.182	0.550
			Early recovery	0.820	1.000
			Mid-recovery	0.267	0.715
			Late recovery	0.787	1.000
		Hardened	Pre-stress	-	-
			1 h stress	0.291	0.756
			3 h stress	0.264	0.713
			6 h stress	0.622	0.989
			Early recovery	0.438	0.916
			Mid-recovery	0.487	0.946
			Late recovery	0.372	0.858
klu	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.212	0.618
			3 h stress	0.413	0.897
			6 h stress	**0.000**	**0.000**
			Early recovery	**0.010**	**0.042**
			Mid-recovery	0.249	0.687
			Late recovery	**0.003**	**0.014**
		Hardened	Pre-stress	-	-
			1 h stress	0.065	0.241
			3 h stress	**0.000**	**0.002**
			6 h stress	**0.036**	0.145
			Early recovery	**0.000**	**0.002**
			Mid-recovery	0.064	0.235
			Late recovery	0.189	0.565
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.987	1.000
			3 h stress	0.217	0.624
			6 h stress	0.332	0.809
			Early recovery	0.468	0.935
			Mid-recovery	0.853	1.000
			Late recovery	0.116	0.388
		Hardened	Pre-stress	-	-
			1 h stress	**0.001**	**0.003**

			3 h stress	**0.003**	**0.015**
			6 h stress	0.132	0.435
			Early recovery	**0.007**	**0.030**
			Mid-recovery	**0.009**	**0.041**
			Late recovery	0.467	0.935
ITP	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.529	0.962
			3 h stress	0.394	0.872
			6 h stress	**0.000**	**0.000**
			Early recovery	0.147	0.461
			Mid-recovery	0.119	0.390
			Late recovery	0.168	0.511
		Hardened	Pre-stress	-	-
			1 h stress	0.692	0.996
			3 h stress	0.795	1.000
			6 h stress	0.623	0.989
			Early recovery	0.019	0.078
			Mid-recovery	0.402	0.885
			Late recovery	**0.032**	0.129
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.107	0.365
			3 h stress	**0.020**	0.083
			6 h stress	0.190	0.568
			Early recovery	0.024	0.099
			Mid-recovery	0.223	0.636
			Late recovery	0.119	0.395
		Hardened	Pre-stress	-	-
			1 h stress	0.467	0.935
			3 h stress	**0.087**	0.311
			6 h stress	0.737	0.999
			Early recovery	0.051	0.196
			Mid-recovery	0.505	0.955
			Late recovery	**0.000**	0.001
sNPF	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.097	0.330
			3 h stress	0.210	0.601
			6 h stress	**0.002**	**0.011**
			Early recovery	0.175	0.527
			Mid-recovery	0.502	0.950
			Late recovery	0.289	0.741
		Hardened	Pre-stress	-	-
			1 h stress	0.302	0.768
			3 h stress	0.564	0.976
			6 h stress	**0.043**	0.168
			Early recovery	**0.043**	0.168
			Mid-recovery	0.105	0.357
			Late recovery	0.530	0.965
	Tropical	Basal	Pre-stress	-	-

			1 h stress	0.053	0.200
			3 h stress	0.060	0.224
			6 h stress	0.101	0.346
			Early recovery	0.116	0.387
			Mid-recovery	0.299	0.763
			Late recovery	0.552	0.972
		Hardened	Pre-stress	-	-
			1 h stress	0.376	0.862
			3 h stress	0.090	0.319
			6 h stress	0.244	0.678
			Early recovery	0.252	0.692
			Mid-recovery	0.310	0.783
			Late recovery	0.073	0.266
Mtl	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.387	0.865
			3 h stress	0.858	1.000
			6 h stress	**0.003**	**0.013**
			Early recovery	0.721	0.998
			Mid-recovery	**0.001**	**0.005**
			Late recovery	0.448	0.917
		Hardened	Pre-stress	-	-
			1 h stress	0.895	1.000
			3 h stress	0.869	1.000
			6 h stress	0.599	0.985
			Early recovery	0.222	0.633
			Mid-recovery	0.411	0.893
			Late recovery	0.080	0.287
	Tropical	Basal	Pre-stress	-	-
			1 h stress	**0.032**	0.128
			3 h stress	0.064	0.235
			6 h stress	**0.045**	0.173
			Early recovery	0.938	1.000
			Mid-recovery	**0.008**	**0.037**
			Late recovery	0.326	0.801
		Hardened	Pre-stress	-	-
			1 h stress	0.650	0.993
			3 h stress	0.372	0.858
			6 h stress	0.488	0.947
			Early recovery	0.981	1.000
			Mid-recovery	0.126	0.419
			Late recovery	0.054	0.204
InR	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.179	0.542
			3 h stress	0.331	0.806
			6 h stress	0.872	1.000
			Early recovery	0.354	0.834
			Mid-recovery	0.462	0.930
			Late recovery	0.313	0.782

		Hardened	Pre-stress	-	-
			1 h stress	0.164	0.515
			3 h stress	**0.001**	**0.006**
			6 h stress	0.130	0.431
			Early recovery	**0.004**	**0.019**
			Mid-recovery	0.093	0.329
			Late recovery	0.108	0.371
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.133	0.432
			3 h stress	0.836	1.000
			6 h stress	0.104	0.356
			Early recovery	0.315	0.786
			Mid-recovery	0.453	0.925
			Late recovery	0.596	0.984
		Hardened	Pre-stress	-	-
			1 h stress	**0.001**	**0.004**
			3 h stress	**0.001**	**0.005**
			6 h stress	0.051	0.197
			Early recovery	0.075	0.273
			Mid-recovery	**0.023**	0.096
			Late recovery	0.070	0.259
FASN2	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.315	0.781
			3 h stress	0.409	0.887
			6 h stress	0.993	1.000
			Early recovery	0.888	1.000
			Mid-recovery	0.052	0.195
			Late recovery	0.893	1.000
		Hardened	Pre-stress	-	-
			1 h stress	0.422	0.902
			3 h stress	0.114	0.384
			6 h stress	0.725	0.998
			Early recovery	0.169	0.520
			Mid-recovery	0.548	0.971
			Late recovery	0.078	0.279
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.102	0.351
			3 h stress	0.447	0.920
			6 h stress	0.637	0.991
			Early recovery	0.607	0.986
			Mid-recovery	0.622	0.989
			Late recovery	0.464	0.932
		Hardened	Pre-stress	-	-
			1 h stress	0.164	0.513
			3 h stress	**0.044**	0.173
			6 h stress	0.135	0.441
			Early recovery	0.065	0.241
			Mid-recovery	0.433	0.912

			Late recovery	0.262	0.709
Treh	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.168	0.511
			3 h stress	**0.014**	0.059
			6 h stress	0.311	0.773
			Early recovery	0.828	1.000
			Mid-recovery	0.991	1.000
			Late recovery	0.940	1.000
		Hardened	Pre-stress	-	-
			1 h stress	**0.001**	**0.003**
			3 h stress	**0.000**	**0.000**
			6 h stress	**0.011**	**0.047**
			Early recovery	**0.000**	**0.001**
			Mid-recovery	0.284	0.742
			Late recovery	**0.026**	0.107
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.158	0.495
			3 h stress	0.757	0.999
			6 h stress	0.329	0.805
			Early recovery	0.316	0.788
			Mid-recovery	0.599	0.985
			Late recovery	0.869	1.000
		Hardened	Pre-stress	-	-
			1 h stress	**0.008**	**0.035**
			3 h stress	**0.036**	0.143
			6 h stress	0.255	0.698
			Early recovery	0.089	0.315
			Mid-recovery	0.327	0.806
			Late recovery	0.397	0.883
CG7084	Temperate	Basal	Pre-stress	-	-
			1 h stress	0.083	0.289
			3 h stress	0.064	0.231
			6 h stress	0.079	0.278
			Early recovery	0.641	0.991
			Mid-recovery	0.479	0.938
			Late recovery	0.881	1.000
		Hardened	Pre-stress	-	-
			1 h stress	**0.015**	0.064
			3 h stress	**0.000**	**0.002**
			6 h stress	0.151	0.479
			Early recovery	**0.023**	0.094
			Mid-recovery	0.278	0.732
			Late recovery	0.951	1.000
	Tropical	Basal	Pre-stress	-	-
			1 h stress	0.977	1.000
			3 h stress	0.821	1.000
			6 h stress	0.409	0.891
			Early recovery	0.901	1.000

		Mid-recovery	0.428	0.907
		Late recovery	0.343	0.822
	Hardened	Pre-stress	-	-
		1 h stress	**0.042**	0.164
		3 h stress	**0.033**	0.133
		6 h stress	0.513	0.958
		Early recovery	0.699	0.997
		Mid-recovery	0.126	0.420
		Late recovery	0.312	0.786

CHAPTER 4

Discussion

4.1 General

Under current climate models, fluctuations in global temperature and precipitation levels are predicted to increase (IPCC 2014). Understanding the capacity of species to withstand this variability has therefore become of paramount concern. Because assessing the most vulnerable species is not always possible, model organisms such as *Drosophila melanogaster* provide an invaluable tool to understanding the mechanisms that might allow species to overcome the deleterious effects of climate change. I took advantage of *D. melanogaster's* natural global distribution to determine the extent to which populations in this species use phenotypic plasticity in response to environmental stress, and then attempted to link these responses with transcriptomic levels responses.

In my first empirical chapter (Chapter 2) I showed that while all quantitative traits exhibited plastic responses to developmental temperature, there was no population differentiation in the extent of the plastic response. That is, both the temperate and tropical population showed similar plastic responses to developmental temperature at the phenotypic level. I also found evidence for plasticity in the transcriptional response of the candidate genes to developmental temperature; overall these plastic responses were larger at the transcript level compared to the quantitative traits level. There was no evidence to suggest that there were genetically based differences in the plastic transcriptional responses. Thus overall, the results of Chapter 2 suggest that although plastic responses at the trait and transcript level are common, there was little evidence for genetically based differences in thermal plasticity.

My second empirical chapter (Chapter 3) focussed on plastic responses to desiccation stress at the transcript and quantitative trait level. In contrast to the results from Chapter 2, I found significant population level divergence in plasticity for desiccation resistance; the tropical population showed a larger plastic response to desiccation stress than the temperature population. However these population level differences were not apparent at the transcript level; while 10 out of the 12 transcripts examined showed plastic responses to desiccation stress, the responses were not population specific.

4.2 Future Directions

4.2.1 Whole transcriptome responses to environmental stress

Trying to determine whole species' potential to counteract the negative impacts of climate change is highly complex. Because of this, many studies have focussed on small pieces of this much bigger puzzle. For example, since the first papers characterising the molecular heat shock response (Lindquist 1981, 1986; Yost & Lindquist 1986), researchers have endeavoured to understand molecular responses to climate stress and how they affect other biological processes such as thermotolerance (Carmel *et al.* 2011), invasion biology (Yu *et al.* 2012) and splicing (Shin *et al.* 2004). The question arises whether we can adequately understand genetic adaptation to environmental stress at the whole organism level by examining only small components in isolation? With the advancement of new techniques such as next-generation sequencing, it is possible to compare the response of multiple populations at the whole genome and transcriptome level. While recent work by Telonis-Scott et al. (2013) has sought to address this deficit by analysing whole genome heat stress responses in a single population of *D. melanogaster*, no studies have yet attempted to understand intra-specific differences in phenotypic plasticity at a genome-wide/transcriptome level in animal models.

Given the complexity of the molecular response to desiccation stress, and to a lesser extent temperature stress, previous studies (Telonis-Scott *et al.* 2012; Telonis-Scott *et al.* 2016; Telonis-Scott *et al.* 2013) have found that whole genome approaches would likely be more useful at elucidating the mechanisms underpinning plasticity in quantitative traits. The third chapter of this thesis uncovered large phenotypic differences in plasticity in response to desiccation stress, but these responses where not replicated at the candidate gene level. With the significant genotype-by-environment interaction observed phenotypically, we know that the difference in the capacity of these populations to mount plastic responses has a genomic basis. However, despite carefully selecting candidate genes that have proven effects on desiccation tolerance, I still found limited evidence for their involvement in maintaining plasticity differences at the phenotypic level. Had I been able to harness the power of RNA-sequencing technology, I suspect I would have found many hundreds, if not

thousands, of genes of small effect contributing to the phenotypic differences that I characterised (Telonis-Scott *et al.* 2016).

While there are examples in the literature of whole-transcriptome responses to various environmental stresses, (for example heat (Telonis-Scott *et al.* 2013), cold (Moskalev *et al.* 2015)) there is a distinct lack of data concerning plastic responses. Given that plasticity plays an important role in species' adaptation to environmental change (Sgrò *et al.* 2016), understanding this responses at a molecular level seems like the logical 'next step'.

4.2.2 Simultaneous stress responses

It goes without saying that climate change does not happen within a vacuum. It affects multiple environmental variables, each affecting multiple traits important to organismal (IPCC 2014). With that in mind, there needs to be a shift from studying single environmental stressor in isolation, to assessing the impacts of multiple stressors on species' responses to climatic change. In particular, temperature and precipitation are intricately connected, and it is likely that the phenotypic and molecular responses described in chapters two and three would be different had they been examined following a combined stress event. For example, Kellermann *et al.* (2012) found that heat resistance increased as precipitation level decreased in *Drosophila spp.* Therefore, high temperature is not the only driver of upper thermal limits, with water in the environment also playing an important role. Furthermore, Bubliy *et al.* (2012) found that temperature and humidity levels play an important role in survival. Flies subjected to both high temperature (37^{o}C) and low humidity (< 10% RH) survived for a significantly shorter time compared to those exposed to high temperature and high humidity (> 90% RH).

My results from chapter three show that temperate flies are more desiccation resistant, but would this be the case if they were simultaneously exposed to high temperature? Previous work using similar populations has found that temperate flies are less heat tolerant than their tropical counterparts (Cockerell *et al.* 2014; Sgrò *et al.* 2010; Telonis-Scott *et al.* 2014). Would the high heat tolerance in tropical flies outweigh the

effect of desiccation stress? Or would the increased desiccation tolerance exhibited by temperate flies result in them surviving longer despite the addition of high heat?

4.2.3 Entire range analyses

My two empirical chapters have employed a cline-end approach to examining climatic adaptation. This method is underpinned by the (empirically supported) assumption that the examined traits are part of a continuous cline, where mean trait values differ predictably along a latitudinal gradient. While this is a good starting point, the next important step would be to undertake these experiments using many more populations sourced along the cline. This is true of the molecular work described in both chapter two and three, and the phenotypic work of chapter three (see Sgro *et al.* (2010) for a comprehensive assessment of the east Australian thermal cline).

The lack of genotype-by-environment interactions observed in the molecular analyses in both of my empirical chapters could be a result of a lack of statistical power. For example, work by Sgro *et al.* (2010) found that hardened heat resistance in *D. melanogaster* followed a negative association with latitude across 17 populations however; when this was repeated four years later (*D. melanogaster* collected in 2008 vs. 2012) using only three populations, no such association was observed (Telonis-Scott *et al.* 2014). While the three populations were derived from distinct latitudinal areas (both cline ends and a mid-range), three data points may be too few to detect any clinal patterns in hardened heat tolerance. Likewise, the two populations that I used undoubtedly resulted in weaker responses and made detecting small geographic differences in transcriptional responses to thermal and desiccation stress less likely.

4.3 Conclusion

In conclusion, the results of this thesis have expanded our understanding of plastic responses to thermal and desiccation stress at the quantitative trait and transcript level across climatically diverse populations of *D. melanogaster*. The research provides insight into how key life history and stress resistance traits respond to thermal and desiccation stress and how such responses may be mediated by transcriptional plasticity. However it is clear that much needs to be done to better our understanding of the molecular basis of plastic responses to climatic stress, and how such responses

may mediate evolutionary responses to on-going climate change not only in *Drosophila* but in other ectothermic species.

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C'est le temps que tu as perdu pour ta rose qui rend ta rose importante.

- Antoine de Saint-Exupéry, *Le Petit Prince*

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Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts

A. S. CLEMSON, C. M. SGRÒ & M. TELONIS-SCOTT

School of Biological Sciences, Monash University, Clayton, Vic., Australia

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gene transcripts;
quantitative traits;
reaction norms;
thermal stress.

Abstract

The flexibility afforded to genotypes in different environments by phenotypic plasticity is of interest to biologists studying thermal adaptation because of the thermal lability of many traits. Differences in thermal performance and reaction norms can provide insight into the evolution of thermal adaptation to explore broader questions such as species distributions and persistence under climate change. One approach is to study the effects of temperature on fitness, morphological and more recently gene expression traits in populations from different climatic origins. The diverse climatic conditions experienced by *Drosophila melanogaster* along the eastern Australian temperate-tropical gradient are ideal given the high degree of continuous trait differentiation, but reaction norm variation has not been well studied in this system. Here, we reared a tropical and temperate population from the ends of the gradient over six developmental temperatures and examined reaction norm variation for five quantitative traits including thermal performance for fecundity, and reaction norms for thermotolerance, body size, viability and 23 transcript-level traits. Despite genetic variation for some quantitative traits, we found no differentiation between the populations for fecundity thermal optima and breadth, and the reaction norms for the other traits were largely parallel, supporting previous work suggesting that thermal evolution occurs by changes in trait means rather than by reaction norm shifts. We examined reaction norm variation in our expanded thermal regime for a gene set shown to previously exhibit GxE for expression plasticity in east Australian flies, as well as key heat-shock genes. Although there were differences in curvature between the populations suggesting a higher degree of thermal plasticity in expression patterns than for the quantitative traits, we found little evidence to support a role for genetic variation in maintaining expression plasticity.

Introduction

Phenotypic plasticity is the ability of a single genotype to generate diverse phenotypes in response to environmental variation (West-Eberhard, 2003). This flexibility is widespread and is predicted to be adaptive when the altered phenotype parallels the native optimum phenotype, but how plasticity impacts adaptive evolution is debatable because of limited empirical data (Ghalambor *et al.*, 2007). Temperature is a ubiquitous factor affecting organismal fitness and distributions, which are often limited to specific thermal ranges to maintain biochemical stability and metabolic activity (David & Tsacas, 1981; Cossins & Bowler, 1987; Hochachka & Somero, 2002). This is particularly true for ectotherms, as their thermal environment dictates the maintenance of homeostasis, body temperature, adult size and ultimately fitness (Huey, 1982; Stevenson, 1985; Angilletta & Dunham, 2003; Angilletta, 2009). The impact of enzyme thermodynamics on thermal sensitivity underpins opposing hypotheses on the evolution of optimal phenotypes in warm- and cold-adapted organisms where it is proposed that 'hotter is better', because

Correspondence: Marina Telonis-Scott, Rm440a, 18 Innovation Walk, Wellington Rd, Clayton, Vic. 3800, Australia.
Tel.: +61 3 9902 4607; fax: +61 3 9905 5613;
e-mail: marina.telonisscott@monash.edu

higher temperatures expedite chemical reactions (Angilletta *et al.*, 2010). The interplay between temperature and plasticity is highly topical in thermal biology research, with a focus on understanding the mechanisms of plasticity and role in phenotypic evolution, population/species diversity and distributions, and persistence in a changing climate (reviewed in Sgrò *et al.*, 2016).

Thermal reaction norms are typically used to describe and compare the effects of temperature (Huey & Stevenson, 1979; Scheiner, 1993) and are a useful measure of the scale and direction of a plastic response. While reaction norms describe the effect of temperature on final trait values, a second measure of thermal plasticity, performance during thermal exposure, can be illustrated by the thermal performance curve (Kingsolver *et al.*, 2004; Angilletta, 2009). The properties defined by the performance curve permit biologically important inferences regarding the thermal optimum (T_{opt}), the temperature at which performance is maximal (P_{max}), and performance breadth and thermal limits (Angilletta *et al.*, 2002). These graphical and mathematical models can be applied empirically to study the evolution of thermal plasticity using different approaches (summarized in Fragata *et al.*, 2015). A common approach in ectotherms is to compare developmental acclimation across a thermal range in populations/species from different climatic origins (Trotta *et al.*, 2006; Yamahira *et al.*, 2007; Liefting *et al.*, 2009; Berger *et al.*, 2013; Klepsatel *et al.*, 2013a; Fallis *et al.*, 2014; Phillips *et al.*, 2014; Zhao *et al.*, 2015). In this framework, differences in the slopes and/or thermal breadth and optima of reaction norms may provide evidence for geographic (i.e. genetic) variation in the direction and/or the magnitude of plasticity (Kingsolver *et al.*, 2004). Similarly, nonadditive effects of the genotype in different environments known as genotype-by-environment interactions (G × E) can indicate genetic variation for plasticity (Scheiner, 1993; Price *et al.*, 2003; DeWitt & Scheiner, 2004).

Drosophila melanogaster is ideal for studying thermal adaptation; this species is viable across a wide temperature range (reviewed in Hoffmann, 2010) and exhibits parallel clines in quantitative fitness and morphological traits, chromosome inversions, DNA polymorphisms, gene expression and other traits (David *et al.*, 1977; James *et al.*, 1997; Azevedo *et al.*, 1998; Land *et al.*, 1999; Gibert & Huey, 2001; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015). Clinal patterns may arise where there are spatially continuous changes in traits, and taking population structure into account, can reflect natural selection to climatic conditions such as temperature (Endler, 1977; Hoffmann & Weeks, 2007). The eastern Australian temperate-tropical latitudinal gradient is an excellent resource to study intraspecific local adaptation given the diverse local climates (Hoffmann & Weeks, 2007), clines in thermal tolerance (Hoffmann *et al.*, 2002; Sgrò *et al.*, 2010) including a thermal candidate gene *Hsrω* (Cockerell *et al.*, 2014), gene expression (Lee *et al.*, 2011; Telonis-Scott *et al.*, 2011) and thermal phenotypic plasticity (Sgrò *et al.*, 2010; Telonis-Scott *et al.*, 2011). Rapid latitudinal shifts in DNA polymorphisms on the Australian east coast may also serve as indicators for climate change (Umina *et al.*, 2005).

Drosophila thermal plasticity research more generally has focused on quantitative phenotypes including morphometrical traits such as bristle number, body size, body colouration and ovariole number (Delpuech *et al.*, 1995; Morin *et al.*, 1999; Moreteau *et al.*, 2003; Gibert *et al.*, 2004; Klepsatel *et al.*, 2013a), and fitness traits such as thermal performance for fecundity (Klepsatel *et al.*, 2013a) and impacts of thermal regime on thermotolerance (Hoffmann & Watson, 1993; Overgaard *et al.*, 2011). Recent high-throughput –omics platforms assess tens of thousands of transcript-level phenotypes simultaneously, and there has been increasing interest in genome-wide thermal expression plasticity correlated with geographic origin and in the role of spatially varying selection in maintaining transcriptome-level variation between populations and species (Levine *et al.*, 2011; Zhao *et al.*, 2015). Genomewide reaction norms have also been used to identify genes with common regulatory architecture and functional roles (Chen *et al.*, 2015).

Thermal variation is also thought to impact performance; Levins (1968) proposed that widespread species experience greater thermal heterogeneity than restricted species, leading to predictions of broader performance breadth in temperate verses tropical *Drosophila* (Overgaard *et al.*, 2011). The limited empirical data, however, is inconclusive; several studies show that while *Drosophila* quantitative traits are highly plastic, differences in some fitness traits are driven by trait mean values rather than differences in reaction norms (plasticity) (Hoffmann & Watson, 1993; Delpuech *et al.*, 1995; Overgaard *et al.*, 2011; Cooper *et al.*, 2012; Klepsatel *et al.*, 2013a). Conversely for morphology, plasticity may be a factor underpinning differences between tropical and temperate *Drosophila* in traits such as size colouration (David *et al.*, 1997; Morin *et al.*, 1999). Molecular phenotypes are also highly plastic; developmental acclimation impacted over 80% of the expressed genes over a broad thermal range in inbred *D. melanogaster* adults (Chen *et al.*, 2015). However, comparative thermal plasticity expression data in outbred populations from different climatic origins is so far limited to two extreme rearing temperatures (Levine *et al.*, 2011; Zhao *et al.*, 2015). There is evidence, however, for G × E for a number of genes suggesting the maintenance of genetic variation for thermal plasticity related to latitude in *D. melanogaster* (Levine *et al.*, 2011; Zhao *et al.*, 2015), but to a lesser extent in *D. simulans* (Zhao *et al.*, 2015). However, the limited number of thermal environments used in these studies provides limited

insight into the relative contribution of plasticity vs. trait mean divergence in climatic adaptation (Sgrò *et al.*, 2016).

In this study, we utilize the well-established 'cline-end' sampling strategy (e.g. Hoffmann & Watson, 1993; Morin *et al.*, 1999; Trotta *et al.*, 2006; Levine *et al.*, 2011) to comprehensively survey thermal plasticity across a wide range of temperatures in a tropical and temperate population of *D. melanogaster* from eastern Australia. Reaction norm variation in quantitative and molecular traits across several thermal environments has not been well studied, and we address this by assessing a test set of 28 fitness, morphological and molecular traits in outbred populations from opposing ends of the same climatic gradient and ask whether mean performance and reaction norms differ according to climatic origin. For the fitness trait fecundity, we examined key parameters of thermal performance, thermal optima, maximum output and breadth. We examined stress resistance variation using standard measures of heat and cold tolerance and examined egg-to-adult viability and body size reaction norms. Utilizing our wider thermal range, we also examined a test set of genes identified from whole transcriptome studies that have previously shown evidence for geographic and/or G × E for thermal plasticity (Levine *et al.*, 2011; Chen *et al.*, 2015; Zhao *et al.*, 2015) to explore potential patterns of spatial selection maintaining genetic variation for molecular plasticity in a comparative framework.

Materials and methods

Drosophila melanogaster collection and maintenance

Drosophila melanogaster were collected using banana baits from Melbourne (temperate; 37.8136°S, 144.9631°E) and Innisfail (tropical; 17.5236°S, 146.0292°E), Australia, in March and May 2013, respectively. From each collection site, 30 wild females were set up in the laboratory as separate isofemale lines. At generation F_2 of laboratory culture, mass-bred populations were established by pooling 10 virgin males and females from each isofemale line (600 flies per population) into two 250-mL bottles containing potato–dextrose–agar medium. The populations were expanded and maintained in sizes of at least 1000 individuals at 25 °C under 12 : 12-h light:dark cycle for 7–21 generations before transfer to the six thermal regimes (Table S1).

Thermal regime experimental design

The experimental populations were initiated at 25 °C in bottles containing standard medium (described above) by placing approximately 250 flies per bottle and allowing females to oviposit for two hours prior to removal of all adults. The bottles were then placed into one of six environmental chambers (MLR-325H; Panasonic, Kadoma, Japan) set to 12 : 12-h light:dark at 16 °C, 18 °C, 22 °C, 25 °C, 28 °C and 30 °C. The developmental temperatures were chosen to represent the range of temperatures that *D. melanogaster* experience in their thermal range permissible to reproduction and development (David *et al.*, 1997). Three bottles per population were placed into each cabinet. Oviposition was staggered across several days to synchronize eclosion based on previous assessment of development rates at the different temperatures, thus permitting simultaneous assessment of all population/temperature combinations.

Quantitative trait phenotyping

Fecundity

Daily female fecundity was examined over a 10-day period. The flies were cultured as for the thermal tolerance assays (described below); however, imagoes were collected and sorted by sex while still virgin. Thirty pairs of female and male flies from each thermal regime from each population were then placed into individual vials with medium and mated for 24 h prior to the commencement of the experiment. Each day, the pairs were aspirated into a new vial containing a spoon with blue-dyed medium and 10-μL activated yeast paste (1 : 3 live yeast:water). The number of eggs each female laid per 24-h period was recorded. Absolute fecundity was determined to be the mean cumulative number of eggs each female laid.

Heat knockdown assay

Heat knockdown time (Hoffmann *et al.*, 2002) was used to assess thermotolerance in 4- to 5-day-old females. Imagoes were collected into mixed-sex cohorts and mated for at least 48 h. At 3–4 days post-eclosion, females were separated into groups of 20 using aspiration without CO_2. The females were maintained in 10-dram vials with medium at their respective developmental temperatures prior to the heat assay. Immediately before the assay, the vials were moved to room temperature and individual females were aspirated into 5-mL glass vials and then immersed in a preheated water bath set to 38.5 °C. Heat knockdown was scored to the nearest second when the fly had become incapacitated. Approximately 30 flies from each population/temperature combination were scored across three replicate assays (blocks), each with approximately 10 flies per population/temperature.

Chill coma recovery

We assessed cold tolerance in 4- to 5-day-old females using a chill coma recovery assay (Gibert *et al.*, 2001). Flies were reared and prepared as for heat knockdown. For the assay, individual females were transferred into empty 1.7-mL Eppendorf tubes and immersed in a

prechilled 0 °C glycol bath and exposed for 6 h. Flies were then removed and allowed to recover at 25 °C, where the time taken (in seconds) for each fly to right itself (stand upright) was recorded. Flies that had not recovered at three hours post-stress were excluded from the analysis (four flies). Approximately 30 flies from each population/temperature combination were assessed simultaneously.

Egg-to-adult viability

Egg-to-adult viability at each of the six developmental temperatures was determined for each population. At 25 °C, approximately 1000 flies were placed onto Petri plates containing medium and *ad libitum* yeast paste and females were allowed to oviposit for two hours. Twenty eggs were then transferred into vials containing medium, and 15 vials were set up per population/temperature combination. As progeny eclosed, they were counted and collected into vials containing medium. At 4–5 days, the females were frozen and stored at −20 °C for the body size measurements.

Body size

Wing size was calculated as a proxy for body size (David *et al.*, 1997). The right wing from 600 females (50 per population/temperature) was removed using forceps, mounted onto a glass slide with double-sided tape and secured with a cover slip. Where the right wing could not be mounted, the left was used instead. Each wing was then photographed using a Leica M80 stereo microscope (Leica, Heerbrugg, Switzerland) with a digital camera attached. Eight wing vein landmark positions were obtained (Fig. S1), and their *x*- and *y*-coordinates determined using tpsDIG software version 2.17 (Rohlf, 2006). Wing area was then measured as centroid size (the square root of the sum of the squared distances from each landmark to the centroid) and calculated using CoordGen8 software (Sheets, 2003).

Quantification of transcript abundance

Candidate gene rationale

To examine the impact of thermal regime and population of origin on molecular phenotypes from the extremes of the same latitudinal gradient, we chose 18 genes according to the following criteria: (i) involvement in thermal tolerance [*Hsf, Hsrω* and *Hsp70Aa* (Hoffmann *et al.*, 2003)] (ii) evidence of population-specific expression variation [*Cyp6g1*, *CG9509* and *CG7214* (Hutter *et al.*, 2008)] and evidence of G × E for expression [temperature-by-populations interactions; *Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B*, *CG6912*, *CG10910*, *CG30083*, *CG33346* and *CG42807* (Levine *et al.*, 2011)]. Transcripts of interest were chosen based on published literature at the study outset; that is, Chen *et al.* (2015) and Zhao *et al.* (2015) had not been published but were incorporated into the cross-study comparison *post hoc*. We examined the multiple isoforms of *Hsf* and *Hsrω* in more detail given the evidence of isoform-specific thermal and/or population responsiveness (Fujikake *et al.*, 2005; Johnson *et al.*, 2011; Lakhotia, 2011; Cockerell *et al.*, 2014). We designed primers to target a common region of all *Hsf* transcripts as well as four isoform-specific primer sets to partition expression of *Hsf-RA*, *RB, RC* and *RD*. The *Hsrω* locus produces multiple nuclear and cytoplasmic long noncoding RNAs, and we examined the longer nuclear transcripts as an isoform subset separately to the shorter cytoplasmic subset. A total of 23 transcripts/transcript subsets were examined in 18 genes. Primer sequences were designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye *et al.*, 2012; Table S2).

Fly collection, mRNA extraction, cDNA synthesis and real-time PCR

The flies for the gene expression assays were collected, sorted by sex and maintained as described for the thermotolerance assays. At day 4–5 post-eclosion, groups of 20 female flies were transferred into 1.7-mL Eppendorf tubes, immediately snap-frozen in liquid N_2 and stored at −80 °C. Five replicates from each population/temperature combination were collected (60 samples in total).

mRNA was isolated from pools of 20 females per sample using a Dynabeads® mRNA DIRECT™ Purification kit (Life Technologies, Carlsbad, CA, USA). Concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and integrity was assessed using 2% agarose gel electrophoresis. cDNA was synthesized in a 20 μL volume from 50 ng mRNA using a Transcriptor High Fidelity cDNA Synthesis kit (Roche, Basal, Switzerland) according to the manufactures' instructions. Two kits were used from separate batches, and the samples reverse-transcribed from each kit were recorded and incorporated into the statistical analyses. The above steps were performed on small, randomized batches of samples. Real-time PCR was performed in 384-well plates using a *Roche* Lightcycler® 480 and SYBR Green chemistry in a 10 μL reaction. Transcripts were amplified using Lightcycler® 480 SYBR Green 1 master-mix where each well contained 5 μL PCR buffer, 4 μL 1 μM primer mix and 1 μL 1 : 9 diluted cDNA. Samples were quantified in duplicate (technical replicates), with five biological replicates analysed per population/temperature combination, except for *Cyp6g1* and *Lectin-galC1*-which were analysed using three biological replicates. Each biological replicate containing all population/temperature combinations was run together on a plate along with three 'housekeeping' genes, *RpL11, Gapdh2 and CycK*. Each housekeeper was verified for both population and thermostability prior to the gene expression assays using two-way analysis of variance (ANOVA; data

not shown). Transcript abundance was quantified relative to the geometric mean (GM) of the housekeepers using the formula: transcript of interest (TOI) = $2^{(GM-TOI)}$.

Reaction norms and performance curves analyses

Fecundity data were analysed as thermal performance curves by assessing BIC of Gaussian functions (Angilletta, 2006) fitted to fecundity (F) in the form:

$$F = a \exp\left[-0.5\frac{(T-b)^2}{c^2}\right]$$

where a is maximum fecundity (u_{max}), b is optimal temperature (T_{opt}), c is the standard deviation of the mean (performance breadth: $T_{br} = 2c$), and T denotes a given experimental temperature. Both thermal performance curves and reaction norm functions were fitted using nls() in R (v3.2.0).

For the heat knockdown, chill coma recovery, viability, body size and transcript abundance data population trait means were related to developmental temperature (i.e. average population-level reaction norms) by fitting first- to fourth-order polynomial functions. Functions with minimal Bayesian information criterion (BIC; Table S3) were selected as best-fitting models (Schwarz, 1978).

Statistical analysis

We next used ANOVA to examine the effects of population and temperature regime on the trait means. Model selection for each trait was determined using diagnostics including Shapiro–Wilk tests for residual normality and Levene's test of equal variances (SAS v9.4, SAS Institute Inc., Cary, NC, USA; PROC UNIVARIATE and GLM, respectively).

For the fecundity data, a mixed model was fit with REPEATED/SUBJECT = individual (population) and the GROUP = temperature statements to account for unequal variances driven by temperature differences (PROC MIXED, SAS V9.4). The fecundity data were also analysed using an analysis of covariance (ANCOVA) with population and temperature as fixed factors and wing size as a covariate. Following Klepsatel *et al.* (2013a,b), fecundity thermal performance curve parameters obtained by fitting Gaussian functions were bootstrapped to determine their standard error. To do this, fecundity data were first simulated based on parameter estimates obtained by fitting Gaussian functions. T_{opt}, u_{max} and T_{br} estimates were then calculated and the process repeated 1000 times for each population.

A fully factorial, two-way general linear-model ANOVA was fit to the heat knockdown and body size data with the fixed effects of population and temperature. A three-way fixed-effects general linear-model ANOVA was fit separately to each gene/transcript, with the fixed effects of cDNA synthesis kit (kit), population and temperature, and two-way interactions between the three main terms. For all transcripts, the effect of kit was stable across the populations and temperatures; therefore, the models were reduced to include only the interaction between temperature and population. Both the heat knockdown and gene expression data were log-transformed to improve normality. The chill coma recovery data were positively skewed and were analysed using a two-way generalized linear model with gamma distribution (link = log). The egg-to-adult viability data analysed using a generalized linear model (link = logit) to account for bimodal distributions with the fixed effects of population and temperature. For the quantitative traits across all temperatures, pair wise-planned contrasts were performed within each population (15 comparisons) and between-population comparisons were performed for the six temperatures (six comparisons) with correction for multiple tests using a false discovery rate (FDR) approach at FDR < 0.05 (Benjamini & Hochberg, 1995).

Finally, we additionally analysed thermal reaction norms for all quantitative and transcript phenotypes (barring fecundity) using either linear or nonlinear regression from the BIC best curve fitting models. For traits with linear reaction norms, linear regression was performed on each population separately with temperature as a continuous factor. For traits with quadratic, cubic or quartic reaction norms, nonlinear regression was performed on the populations separately. Each nonlinear regression had either two, three or four continuous factors (quadratic: temperature and temperature^2; cubic: temperature, temperature^2 and temperature^3; quartic: temperature, temperature^2, temperature^3 and temperature^4). The raw data were fit for each model, and the transcript data were fit with kit as a main factor. The least-squares means (LS means) derived from the full ANOVA models for all traits are shown for illustrative purposes.

Results

Effects of thermal regime on quantitative traits

Mean daily fecundity was significantly affected by temperature, population, and the interaction between them (Table 1). For each population separately, all pairwise temperature comparisons were performed (15 comparisons), but for interpretative ease, between-population comparisons were restricted to the same temperature (six comparisons). Within-population pairwise-planned contrasts were significant for all comparisons except 25 °C vs. 28 °C for both tropical and temperate females (FDR < 0.05, Fig. 1). On average, the tropical females were significantly more fecund at 16 °C, whereas the temperate females were more fecund at all rearing temperatures except 30 °C (FDR < 0.05, Fig. 1). We also

Table 1 Results for two-way ANOVAs on the on the fixed effects of developmental temperature, population (temperate and tropical) and the interaction term for fecundity*, heat knockdown time and body size. Significant terms are shown in bold.

Trait	Source of variation	d.f.	SS	*F*	*P* value
Fecundity	Temperature	5	–	3802.7	**1E-15**
	Population	1	–	7.52	**0.006**
	Temperature × Population	5	–	23.6	**1E-15**
	Error		–		
Heat	Temperature	5	39.832	72.06	**1E-15**
	Population	1	0.421	3.81	**0.052**
	Temperature × Population	5	0.812	1.47	0.199
	Error	344	38.028		
Body size	Temperature	5	14.081	1304.74	**1E-15**
	Population	1	0.432	199.81	**1E-15**
	Temperature × Population	5	0.013	1.25	0.287
	Error	588	1.27		

*Fecundity data were fit using a mixed-model ANOVA to account for unequal variances and uses a likelihood-based estimation where sum of squares (SS) are not output.

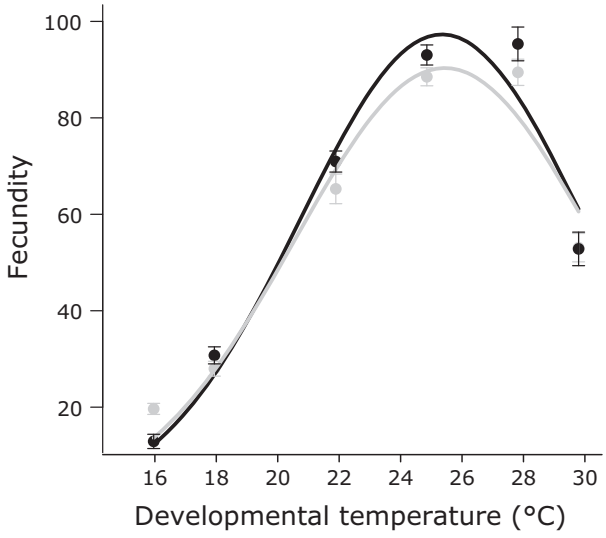

Fig. 1 Thermal performance curves for average daily fecundity in temperate (black) and tropical (grey) flies. Error bars represent standard error of the least-squares means.

examined the effect of body size on mean daily fecundity using ANCOVA and found no effect of body size, but significant effects of temperature, population and the interaction between them (temperatature: $F_{5,346} = 1252.5$, $P < 0.0001$; population: $F_{1,346} = 7.94$, $P < 0.01$; temperature-by-population: $F_{5,346} = 23.34$, $P < 0.0001$).

Fecundity performance curve parameter estimate analyses showed that the tropical and temperate females did not differ in their optimal temperatures (T_{opt} temperate: 25.5 °C ± 0.12 °C; tropical: 25.59 °C ± 0.14 °C) or performance breadth (T_{br} temperate: 9.33 °C ± 0.28 °C; tropical: 9.86 °C ± 0.32 °C). However, the populations did differ in their maximum fecundity with temperate females producing more eggs on average per day than tropical females (u_{max} temperate: 97.29 ± 2.08 eggs/day; tropical: 90.32 ± 1.87 eggs/day).

Rearing temperature significantly affected trait means for heat knockdown and body size (Table 1), chill coma recovery and egg-to-adult viability (Table 2). Population of origin also significantly impacted mean chill coma recovery time and body size (Tables 1 and 2) and marginally for mean heat knockdown time (Table 1, $P = 0.0518$). The impact of thermal regime on the trait means was similar between the populations, evidenced by the lack of temperature-by-population interactions (Tables 1 and 2) and qualitatively parallel reaction norms (Fig. 2a–d).

For heat knockdown, within-population pairwise-planned contrasts were significant for all comparisons except 16 °C vs. 18 °C, 22 °C vs. 25 °C and 28 °C vs. 30 °C, and the same result was observed in both populations (Fig. 2a. FDR < 0.05 for all other comparisons). Between-population contrasts were significant only at 16 °C and 18 °C due to higher knockdown resistance in temperate females compared to tropical females

Table 2 Results for two-way generalized linear-model ANOVAs on the fixed effects of developmental temperature, population (temperate and tropical) and the interaction term for chill coma recovery time and egg-to-adult viability. Significant terms are shown in bold.

Trait	Source of variation	d.f.	χ^2	*P* value
Cold	Temperature	5	281.3	**1E-15**
	Population	1	17.65	**2.7E-05**
	Temperature × Population	5	7.9	0.162
Viability	Temperature	5	31.88	**6.3E-06**
	Population	1	0.68	0.41
	Temperature × Population	5	7.05	0.217

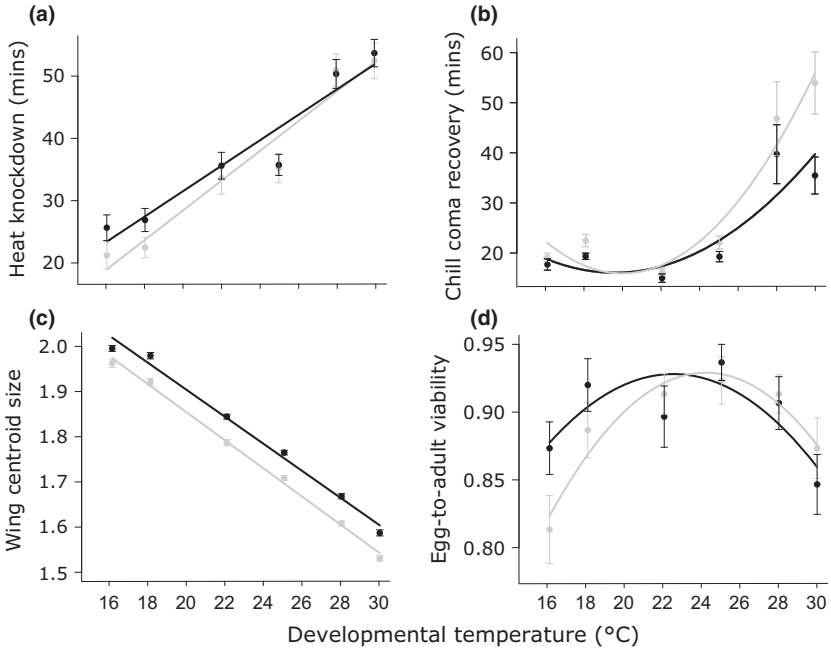

Fig. 2 Thermal reaction norms for quantitative trait population means in temperate females (black) and tropical (grey) females from eastern Australia. (a) Linear reaction norms for heat knockdown resistance, (b) quadratic reaction norms for chill coma recovery, (c) linear reactions norms for body size (approximated via wing centroid size) and (d) quadratic reaction norms for egg-to-adult viability. Error bars represent standard error of the least-squares means.

(Fig. 2a. FDR < 0.05 and < 0.1, respectively). For chill coma recovery, rearing temperature reduced recovery time at the high temperature extremes; within-population pairwise-planned contrasts were significant for contrasts except 16 °C vs. 18 °C, 16 °C vs. 22 °C, 16 °C vs. 25 °C, 18 °C vs. 25 °C and 28 °C vs. 30 °C (Fig. 2b. FDR < 0.05 for all other comparisons). The temperate females were more chill coma resistant than tropical females only at 30 °C (FDR < 0.05, Fig. 2b). Within populations, body size was significantly different between all temperatures except 16 °C vs. 18 °C, and the mean body size of the temperate females was consistently larger than the tropical females across the thermal range (FDR < 0.05, Fig. 2c). Egg-to-adult viability was less variable within populations; tropical flies were less viable at 16 °C compared to 22 °C, 25 °C and 28 °C and at 16 °C vs. 25 °C for the temperate population, whereas viability was higher in temperate flies at 18 °C vs. 30 °C and 25 °C vs. 30 °C (FDR < 0.05, Fig. 2d). There were no between-population differences at each temperature.

We also analysed thermal reaction norms using linear and nonlinear multiple regressions. Temperature had a significant positive linear relationship with heat knockdown time in both populations (Table S4), where knockdown resistance improved with increasing rearing temperature (Fig. 2a). Reaction norms for chill coma recovery in both populations were negative quadratic, with significant main effects of temperature and temperature^2 (Fig. 2b, Table S4). There was a strong negative linear relationship between temperature and body size, where size decreased with increasing rearing temperature (Fig. 2c, Table S4). Egg-to-adult viability reaction norms were quadratic, with the parabola concave downwards (i.e. 'bell' shaped, Fig. 2d), and although both temperature and temperature^2 main terms were significant, the overall model explained very little of the variation in viability (Table S4).

Effects of thermal regime and genotype on transcript-level phenotypes

Similar to the quantitative traits, rearing temperature had the most significant effect on mean transcript expression (22/23 transcript, three-way ANOVA, Table S5). Transcript abundance differed between the populations for 15/23 transcripts (Table S5), with a bias towards higher mean expression in tropical females (12/15 transcripts, Fig. 3). There was little evidence of G × E for expression variation; only two transcripts had significant temperature-by-population interaction terms (*Hsf-RA* and *mag*, $P < 0.05$ and 0.01, respectively, Table S5); however, these terms did not remain significant following FDR correction. For brevity, we restrict our results to description of reaction norms and not planned contrasts of means within and between populations.

Negative linear expression reaction norms in both populations

We observed an array of gene expression reaction norms fitting to first- to fourth-order polynomial functions (Table S3). For each transcript and population, linear and nonlinear multiple regressions were performed with the main terms fit after choosing the order of the reaction norm based on the BIC best-fitting

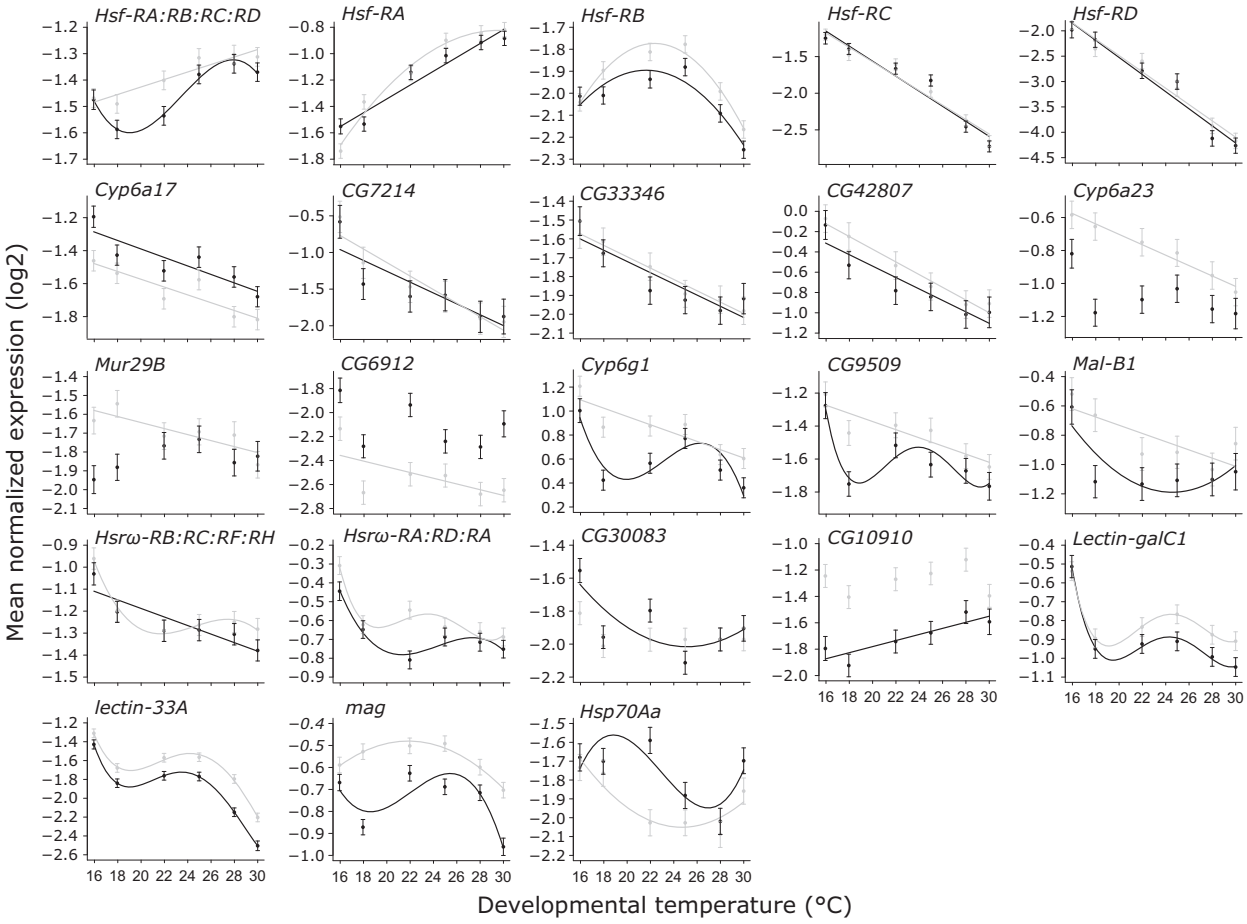

Fig. 3 Gene expression thermal reaction norms in temperate (black) and tropical (grey) flies for 23 mRNA transcripts (18 genes). Error bars represent standard error of the least-squares means.

models (Fig. 3; Table S6). For six transcripts, the main effect of temperature was significant in the linear regression in both the tropical and temperate populations (*Hsf-RC, Hsf-RD, Cyp6a17*, *CG7214*, *CG33346*, *CG42807*, Table S6, Fig. 3). The populations were invariant in trait means for the latter transcripts except *Cyp6a17* (ANOVA, Table S5), and the reaction norms were negative linear, that is expression decreased with increasing rearing temperature (Table S6, Fig. 3).

Population-specific, negative linear and nonlinear expression reaction norms

For three genes *Cyp6a23*, *Mur29B* and *CG6912*, the expression reaction norms were significantly negative linear in the tropical population only, whereas the main term of temperature was not significant in the temperate population (Table S6, Fig. 3). For three genes *Cyp6g1*, *CG9509* and *Mal-B1*, the reaction norms were negative linear in the tropical population and negative quadratic in the temperate population (Table S6, Fig. 3). Temperature had a significant quadratic relationship with expression (decreasing with slight U shape) in temperate females for *CG30083*, but no main terms were significant in the tropical population (Table S6, Fig. 3).

The shape of the curves was also differentiated between the two populations for the *Hsrω* transcript subsets; the *Hsrω-RB:RC:RF:RH* temperate population reaction norms were negative linear, whereas the tropical population curve was sigmoid (s-shaped, decreasing with increasing rearing temperature) with significant temperature, temperature^2 and temperature^3 main terms in the multiple regression (Table S6, Fig. 3). Both population reaction norms for *Hsω-RA:RD:RG* were negative sigmoid as for *Hsrω-RB:RC:RF:RH*, with an additional temperature^4 component in the multiple regression in the tropical population (Table S6, Fig. 3).

Population-specific positive linear and nonlinear expression reaction norms

Rearing temperature had a positive linear relationship with expression in both populations for *CG10910*, but

was specific to temperate flies for *Hsf-RA* and tropical flies for *Hsf-RA:RB:RC:RD* (Table S6, Fig. 3). The tropical population *Hsf-RA* reaction norm was better described by a quadratic rather than linear model (Table S6, Fig. 3). Temperate expression of *Hsf-RA:RB:RC:RD* was more complex than the tropical population where expression was lowest at 18 °C and 22 °C and highest at 25–30 °C, resulting in a positive s-shaped curve with significant linear, quadratic and cubic temperature main terms in the multiple regression (Table S6, Fig. 3).

Higher-order nonlinear expression reaction norms

For three transcripts, both populations exhibited similar complex thermal expression curves (*Hsf-RB*, *Lectin-galC1*, *lectin-33A*, Fig. 3). The *Hsf-RB* reaction norms were convex curvilinear (i.e. 'bell' shaped), whereas reaction norms for *Lectin-galC1* and *lectin-33A* were complex, quartic-shaped curves where expression was highest and lowest at the low and high temperature extremes, respectively, with intermediate expression in the mid-temperature range (Table S6, Fig. 3). We also observed population-specific, nonlinear reaction norms for two transcripts, *mag* and *Hsp70Aa*, where the tropical populations exhibited quadratic reaction norms whereas the temperate population reaction norms were higher-order cubic with significant temperature, temperature^2 and temperature^3 main terms in the multiple regression (Table S6, Fig. 3).

Isoform-specific expression reaction norms: Heat-shock factor (Hsf)

Interestingly, separate quantification of the four *Hsf* transcript isoforms revealed not only differences in the effects of thermal regime and population on expression means (ANOVA, Table S5), but also variation in reaction norms that differed from the 'gene-level' reaction norms (Fig. 3). Quantification of *Hsf* expression at the gene level (i.e. *Hsf-RA:RB:RC:RD*) would suggest that expression largely increases with rearing temperature with slightly different curve shapes between the populations, and whereas this is true for one isoform *Hsf-RA*, the *Hsf-RC* and *Hsf-RD* isoform curves were parallel negative linear, whereas for *Hsf-RB,* both populations exhibited bell-shaped reaction norms (Table S6, Fig. 3).

Cross-study comparisons of transcript thermal response means

Given our evidence-based approach in choosing a test set of loci for assessing thermal responses of transcript phenotypes in natural populations of *D. melanogaster* (see Materials and Methods), we next compared our data where possible to that in the literature. For the 18 genes examined here, we documented whether all main terms in the ANOVAS (temperature, population and temperature-by-population interactions) were tested in the other studies, which sex was assessed and genetic background of the populations (inbred or outbred), and where overlap between our study and other studies for a particular term/terms occurred (Table 3). As we undertook a candidate gene approach for 18 genes based on previous findings across a range of full transcriptome studies, we could not statistically quantify the degree of overlap but rather qualitatively report common outcomes (Table 3).

'Core' genes with thermal plasticity/and or geographic variation in diverse Drosophila melanogaster

For thermal plasticity, we observed a high degree of overlap with Chen *et al.* (2015) (15/18 genes, Table 3) and Levine *et al.* (2011) (13/18 genes, Table 3) and next with the outbred north American populations (Zhao *et al.*, 2015) (8/18 genes, Table 3). A core group of four thermally responsive genes were common to all studies: *Cyp6a23, Mal-B1*, *CG7214*, *CG42807* (Table 3). Thermal plasticity of heat-shock-related genes was observed across studies: *Hsrω* (here, Zhao *et al.*, 2015; and Chen *et al.*, 2015; Table 3), *Hsp70Aa* (here and Zhao *et al.*, 2015; Table 3) and *Hsf* (here and Chen *et al.*, 2015; Table 3).

Where we could compare differential expression of genes between populations, we found seven genes overlapped with the north American populations (Zhao *et al.*, 2015; Table 3). Apart from the current study and Levine *et al.* (2011), only Zhao *et al.* (2015) tested for G × E but they found no significant interactions for the 18 genes examined here (Table 3).

Comparison of gene expression reaction norms with other Drosophila melanogaster from the east coast of Australia

The populations studied here, and in Levine *et al.* (2011), were geographically most comparable: here, females from temperate and tropical populations (southern temperate Melbourne and northern tropical Innisfail) were reared at six temperatures, whereas Levine *et al.* (2011) compared males from a more southern temperate population (Tasmania) to tropical Innisfail reared at 18 °C and 30 °C. The latter temperatures were chosen with the rationale that they approximate the average 'home' temperatures naturally experienced by flies from the temperate and tropical populations, respectively (Levine *et al.*, 2011). The authors reported significant temperature effects on the transcriptome, G × E for 56 genes (FDR 0.1) as well as enrichment of 'home and away' directionality of expression (i.e. higher expression in temperate flies reared at 18 °C vs. attenuated expression at 30 °C and *vice versa* in the tropical population).

We examined 12 genes exhibiting G × E in Levine *et al.* (2011), and although temperature significantly impacted all genes and 10 genes were differentially expressed between the populations (Table 3), we found little evidence for G × E or 'home and away' gene

Table 3 Summary of the cross-study comparison for the 18 *Drosophila melanogaster* genes chosen in the current study based on previous evidence of temperature expression plasticity[1,2,3], and/or genotype (geographic) expression variation[1,2] and/or genotype-by-environment interactions (G × E) between population of origin and thermal regime[1,2,3]. Significant genes* in each study are shown for each category (N/A denotes where terms were not assessed in a study).

Study/design	Thermal plasticity	Genotype/geographic variation	G × E	Inbred (I) or Outbred (O)	Sex
Current study EA Cline ends 16 °C, 18 °C, 22 °C 25 °C, 28 °C, 30 °C	*Hsf, Hsrω, Hsp70Aa*, *Cyp6a17, Cyp6a23, Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912, CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	*Hsf*, *Hsrω*, *Hsp70Aa*, *Cyp6a17, Cyp6a23, Cyp6g1*, *Lectin-galC1*, *lectin33A*, *mag*, *Mal-B1*, *Mur29B, CG6912*, *CG9509*, *CG10910*, *CG30083*	*Cyp6a23*†, *mag*†, *Mur29B*†	O	Female
Zhao *et al.* (2015)[1] NA Cline ends 21 °C, 29 °C	*Hsrω*, *Hsp70Aa*, *Cyp6a23*, *Lectin-galC1, Mal-B1*, *CG7214*, *CG9509*, *CG42807*	*Cyp6a23*, *Cyp6g1*, *Lectin-galC1*, *Mur29B*, *CG6912*, *CG9509*, *CG42807*	None	O	Male
Levine *et al.* (2011)[2] EA Cline ends 18 °C, 30 °C	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B, CG6912, CG7214, CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	*Cyp6a17*, *Cyp6a23*, *Lectin-galC1*, *lectin-33A*, *mag*, *Mal-B1*, *Mur29B, CG6912, CG10910*, *CG30083*, *CG33346*, *CG42807*	O	Male
Chen *et al.* (2015)[3] Lab strains 13 °C, 18 °C, 23 °C, 29 °C	*Hsf, Hsrω*, *Cyp6a17, Cyp6a23, Cyp6g1*, *lectin33A*, *mag*, *Mal-B1*, *CG6912, CG7214*, *CG9509*, *CG10910*, *CG30083*, *CG33346*, *CG42807*	N/A	N/A	I	Female

EA, eastern Australia; NA, north America.
*Significance thresholds vary from study to study; we used genes from reported results.
†Significant interaction terms in the ANOVA in the current study that were nonsignificant at FDR 0.05.

expression directionality across six rearing temperatures apart from weak signal at the *mag* locus (ANOVA, Table S5). For a more direct comparison, we next analysed trait means only for 18 °C and 30 °C using three-way ANOVAS. We found less thermal plasticity at 18 °C and 30 °C compared to Levine *et al.* (2011) than across our full thermal range, with only five genes significant for the main effect of temperature (Table S7, Fig. S3). The populations differed in transcript abundance for seven genes, biased to higher expression in the tropical population (Table S7, Fig. S3). Only two genes, *Cyp6a23* and *Mur29B*, had significant gene-by-environment interaction terms, although the direction of expression was opposite to the 'home and away' pattern observed by Levine *et al.* (2011) and similar to *mag* for all six temperatures, although the significance was lost after FDR correction (uncorrected $P < 0.05$, FDR < 0.2, Table S7).

Comparison of gene expression reaction norms with inbred Drosophila melanogaster

Chen *et al.* (2015) classified gene expression reaction norms in inbred female *D. melanogaster* across a broader thermal regime overlapping with the range employed here (ranging from 13 °C to 29 °C). Where possible, we compared the direction of expression plasticity (i.e. increasing or decreasing with temperature) and reaction norm curvature (Table 4). Fifteen genes were comparable between the two studies (Tables 3 and 4); there was concordance for the direction of expression plasticity (10/15 genes, Table 4), with 8/10 genes decreasing in expression with increasing rearing temperature, one gene increasing with rearing temperature and one gene with *U*-shaped expression over the thermal regimes (Table 4). There was also overlap in reaction norm shape (7/15 genes, Table 4) where at least one of the populations here exhibited the same curvature as Chen *et al.* (2015).

Discussion

We compared thermal plasticity between female *D. melanogaster* from the ends of the eastern Australian temperate-tropical latitudinal gradient for 28 phenotypes across six rearing temperatures. Temperature impacted almost every phenotype ranging from quantitative fitness and morphological traits to gene transcripts. Although there were some differences between the populations for quantitative trait means, fecundity thermal performance and reaction norms for thermotolerance, body size and viability were comparable, supporting a view of ectotherm thermal adaptation by shifts in average trait values rather than reaction norm shape (Yamahira *et al.*, 2007; Klepsatel *et al.*, 2013a;

Fragata *et al.*, 2015). The gene expression traits revealed more complexity in response curves between the populations, although we found little evidence for a genetic component underpinning the plasticity variation in contrast to previous findings (Levine *et al.*, 2011).

Similar performance and reaction norm variation in quantitative traits

For reproductive performance measured as absolute fecundity, we found no difference between tropical and temperate thermal optima or performance breadth, in agreement with cross-continent *D. melanogaster* populations (Klepsatel *et al.*, 2013a). The temperate females had higher maximum output, however, consistent with previous *Drosophila* studies which reject the 'hotter is better' hypothesis of performance (Klepsatel *et al.*, 2013a; Fragata *et al.*, 2015). 'Hotter is better' predicts a positive correlation in maximal performance with increased thermal selection, that is higher output from the tropical population (Angilletta *et al.*, 2010). Higher output of temperate flies could result from the positive correlation with body size and fecundity (see Klepsatel *et al.*, 2013b), but we failed to find a relationship despite the larger size of temperate females across the thermal range. Unlike some *D. subobscura* populations, 'bigger wasn't always better' (Fragata *et al.*, 2015); we observed no differences at the highest temperature (30 °C), and the tropical females were more fecund than temperate females at the mildest temperature (16 °C). The latter result is surprising given that larger more cold-adapted flies often perform better at lower temperatures (Reeve *et al.*, 2000; Bochdanovits & De Jong, 2003).

In contrast to our results, Klepsatel *et al.* (2013a) found higher fecundity in temperate *D. melanogaster* at intermediate temperatures whereas tropical females performed better at high temperatures, and in another study measuring reproductive output as productivity, temperate populations did better in the cold but worse in the heat compared to tropical populations (Trotta *et al.*, 2006). It is unclear what factors underlie the differences; here, possible explanations include insufficient power to detect differences using a narrower, constant thermal range in two populations from the same climatic gradient compared to the six cross-continent populations reared under the fluctuating regime employed by Klepsatel *et al.* (2013a). On the Australian east coast, although average minimum temperatures decrease with latitude, it is possible that thermal selection at upper temperatures experienced by the populations studied here may be similar given maximum yearly temperatures are largely uniform, and maximum summer temperatures are similar between tropical Innisfail and temperate Melbourne (Hoffmann, 2010; Overgaard *et al.*, 2011; Kristensen *et al.*, 2015). Average temperatures, however, do not reflect sudden fluctuations in temperature extremes, which are experienced more frequently by temperate eastern Australian flies compared to their tropical counterparts (Hoffmann, 2010). Despite evidence for thermal selection on the Australian east coast in opposing thermotolerance clines (Hoffmann *et al.*, 2002; Sgrò *et al.*, 2010), we found little variation between the populations for chill coma recovery and heat knockdown; rather, the temperate population better resisted heat knockdown at 16 °C and 18 °C and recovered from cold exposure faster at 30 °C. Maintaining higher stress resistance at the ends of the thermal range could reflect a better ability of temperate flies to withstand temperature extremes. In the laboratory, temperate Australian *D. melanogaster* may be physiologically more capable to withstand sudden, extreme

Table 4 Cross-study comparison of *Drosophila melanogaster* gene expression reaction norms for 15 genes assessed here (reared at 16 °C, 18 °C, 22 °C 25 °C, 28 °C, 30 °C) with Chen *et al.* (2015) (reared at 13 °C, 18 °C, 23 °C, 29 °C).

	Curvature (current study)				
Gene	Tropical	Temperate	Curvature (Chen *et al.*, 2015)	Plasticity (current study)	Plasticity (Chen *et al.*, 2015)
Hsf	Linear	Quadratic	Linear	Increasing	Increasing
CG33346	Linear	Linear	Linear	Decreasing	Decreasing
Cyp6a17	Linear	Linear	Quadratic	Decreasing	Decreasing
CG7214	Linear	Linear	Quadratic	Decreasing	Decreasing
CG42807	Linear	Linear	Quadratic	Decreasing	Decreasing
Cypg1	Linear	Quadratic	Linear	Decreasing	Decreasing
CG9509	Linear	Quadratic	Linear	Decreasing	Decreasing
Mal-B1	Linear	Quadratic	Linear	Decreasing	Decreasing
CG6912	Linear	–	Quadratic	Decreasing	Decreasing
CG30083	–	Quadratic	Quadratic	U	U
Cyp6a23	Linear	–	Quadratic	Decreasing	Bell
Mur29B	Linear	–	Quadratic	Decreasing	U
CG10910	Linear	Linear	Quadratic	Increasing	U
Lectin-33A	Quartic	Quartic	Linear	Decreasing	Increasing
Mag	Quadratic	Cubic	Quadratic	Decreasing	Bell

temperature changes than tropical populations (Sgrò *et al.*, 2010), although the extent to which natural extremes are mitigated via behavioural avoidance through habitat selection could be important (Feder *et al.*, 2000).

Despite variations in mean thermotolerance between the populations, the reaction norms were parallel in shape, consistent with observations of similar thermotolerance plasticity between *D. melanogaster* populations (Hoffmann & Watson, 1993; Bubliy *et al.*, 2002; Hoffmann *et al.*, 2005; Cooper *et al.*, 2012) and among widespread and tropical *Drosophila* species (Overgaard *et al.*, 2011). We found that rearing temperature impacted the traits in the direction anticipated, that is increased resistance to heat knockdown with increasing rearing temperature and the opposite for chill coma resistance, consistent with high levels of plasticity for stress resistance in response to environmental conditions (discussed in Hoffmann *et al.*, 2005). The similarity in phenotypic plasticity at the intra- and interspecific level holds independent of thermal regimes (i.e. developmental or short-term acclimation, fluctuating or constant conditions).

For body size, the plastic (i.e. decrease in size with increasing temperature) and genetic responses (consistently larger temperate females) both comprised parallel vertical shifts in the trait means, consistent with most intraspecific comparisons, including continent-wide clinal studies and/or reaction norm analyses (Coyne & Beecham, 1987; James *et al.*, 1995; Land *et al.*, 1999; Trotta *et al.*, 2006), but see Morin *et al.* (1999). We found no population differences in reaction norms for viability, a key indicator of pre-adult fitness, in agreement with European *D. melanogaster* and *D. simulans* populations (Petavy *et al.*, 2001). Similarly, thermal plasticity for viability to alternating regimes was invariant between highland vs. lowland Argentinian populations sampled from opposing latitudinal and altitudinal viability clines (Folguera *et al.*, 2008), in contrast, however, to South American populations that differed slightly in reaction norms, whereas trait values did not vary over latitude (Land *et al.*, 1999). Here, however, the impact of temperature was mild, and viability remained above 80% across the thermal range, similar to observations between 14 and 28 °C in *D. melanogaster* and *D. simulans* (Petavy *et al.*, 2001). Therefore, we did not assess viability performance as a trait given our thermal range did not quite capture the stressful temperatures (particularly at the low end) (Petavy *et al.*, 2001; Kristensen *et al.*, 2015) that might more clearly define performance parameters such as performance breadth.

Expression plasticity

The high degree of thermal plasticity at the transcript level (22/23 transcripts) is unsurprising given our gene selection criteria, with the majority also thermally responsive among different *D. melanogaster* transcriptome studies (Levine *et al.*, 2011; Chen *et al.*, 2015; Zhao *et al.*, 2015). We found four 'core' genes with consistent temperature modulated expression independent of genetic background or study design, suggesting some degree of conserved thermal plasticity in *D. melanogaster*. Chen *et al.* (2015) reported a higher degree of thermal plasticity than previous estimates from fewer temperatures (Levine *et al.*, 2011; Zhou *et al.*, 2012; Zhao *et al.*, 2015), and for our gene set, we observed the most overlap with Chen *et al.* (2015) likely due to the broader thermal regimes employed, where plasticity increases with additional environmental exposures.

Although not as extensive as the effect of temperature, mean expression of two-thirds of the genes differed between the tropical and temperate populations, with higher expression levels predominantly biased to the tropical population. Expression variation within and between populations is well documented (Oleksiak *et al.*, 2002; Michalak *et al.*, 2007; Levine *et al.*, 2011; Müller *et al.*, 2011; Catalán *et al.*, 2012), reflective of ample genetic variation for differential transcript abundance, although the fitness consequences of gene expression divergence remain largely unknown (Feder & Walser, 2005; Evans, 2015). Expression differences often arise from *cis* regulatory variation and also copy number variation: here two differentially expressed examples are *Cypa17* and *Cyp6g1*, confirming previous work (Hutter *et al.*, 2008; Catalán *et al.*, 2012) and copy number variation for these genes are known and related to expression for DDT-like pesticide resistance (Schmidt *et al.*, 2010) which may vary according latitude along the east Australian coast (Turner *et al.*, 2008). Despite the lack of evidence here, there is increasing support for a role of spatially varying selection in maintaining adaptive gene expression variation in diverse environments, where differences in adaptive phenotypes may be evidenced through plasticity (see Levine *et al.*, 2011). G × E interactions maintain variation in plasticity across different genotypes and are prevalent in gene expression data (Levine *et al.*, 2011; Dayan *et al.*, 2015; Zhao *et al.*, 2015), although as above, the contribution of gene expression plasticity to organismal fitness is less clear (Hodgins-Davis & Townsend, 2009), but general patterns can provide broader insight into evolution in heterogeneous environments.

Lack of support for G × E for expression plasticity

We expanded the developmental thermal regime for 12 highly significant genes from previous research reporting expression G × E at 18 °C and 30 °C in eastern Australian *D. melanogaster* (Levine *et al.*, 2011), but failed to replicate G × E or directional 'home and away' expression plasticity of expression trait means, even when compared only 18 °C and 30 °C. One

explanation is study design differences; Levine *et al.* (2011) pooled males from isofemale lines whereas we assessed mass-bred females derived from isofemale lines which may impact gene expression comparisons owing to patterns of linkage disequilibrium and/or sex-specific effects. Further, although the tropical populations were from the same town in both studies, we assessed a temperate southern mainland population whereas Levine *et al.* (2011) compared a temperate population further south from island Tasmania, although Tasmanian *D. melanogaster* are not isolated given the evidence for gene flow between populations (Kennington *et al.*, 2003).

Standing genetic variation in different populations will impact G × E; genes that exhibit differential expression for genotypes in different environments are impacted by local but predominantly upstream regulatory sequence variation (Hodgins-Davis & Townsend, 2009; Grishkevich & Yanai, 2013) and at the genome-wide level may even be categorized as more likely to exhibit G × E by distinctive genomic and structural features (Grishkevich & Yanai, 2013). As such, differences in DNA polymorphisms in the temperate populations examined here, and by Levine *et al.* (2011), compared to tropical Innisfail could be a factor, and we also cannot rule out the impact of seasonal variation on standing genetic variation given the extensive temporal shifts documented in natural *D. melanogaster* (Itoh *et al.*, 2010; Bergland *et al.*, 2014). Perhaps, the highly environmentally plastic nature of the transcriptome (Hodgins-Davis & Townsend, 2009) coupled with genetic shifts from sampling season renders replicable signatures of G × E for gene expression traits difficult between temperate and tropical eastern Australian populations.

Although there was little support for genetic variation in mean expression plasticity between the populations, there were differences in the thermal expression reaction norm curves, suggesting a high degree of population-specific plastic variation in contrast to the largely parallel quantitative trait reaction norms. Differences in the shape of the response curves between populations could be passive responses due to neutral sequence variation, thermal stress or other unknown constraints (Gibert *et al.*, 1998; Levine *et al.*, 2011), or they could be adaptive and therefore useful in identifying putative selection targets (Gibert *et al.*, 1998). The results are promising for future work harnessing the transcriptome as a powerful set of traits and broader thermal regimes to explore the evolutionary basis of plasticity in a modelling framework (e.g. Gibert *et al.*, 1998). Although the reaction norms were surprisingly dissimilar with previous eastern Australian *D. melanogaster* (Levine *et al.*, 2011), we found repeatability in the direction of plasticity and curvature in at least one of our populations with Chen *et al.* (2015), highlighting common expression responses to a broader thermal range, but whether the same mechanisms underpin the trait responses remains an open question. It is worth noting that three of the five genes (*Cyp6a23*, *mag* and *Mur29B*) that exhibited different reaction norms between the populations here and also compared to Chen *et al.* (2015) exhibited weak evidence for G × E in the current study (either across the entire thermal range or at 18 °C and 30 °C), suggestive of segregating genetic variation for plasticity in these genes, although more population data are required to further explore this.

Further complexity of transcript reaction norm variation: *Hsf*

Finally, in addition to testing genes with previous population-level expression differentiation and/or population-by-temperature interactions, we examined plasticity in three key genes involved in the heat-shock response, *Hsp70Aa*, *Hsrω* and the master regulator *Heat-shock factor* (*Hsf*). Chen *et al.* (2015) also reported reaction norms for a number of *Hsps,* and similar to here, the genes do not always show a clear relationship with temperature for this thermal range. Although we found differences in thermal regulation of the genes, the thermal plasticity appears not to have a discernible genetic component, and in combination with the largely similar thermotolerance phenotypes, data suggest a lack of divergence in these populations. Linking heat-shock genes to adaptive thermotolerance is problematic, however (Telonis-Scott *et al.*, 2014), but what was striking here was the complexity of reaction norms for the isoforms of *Hsf* compared to the gene-level reaction norms. Previously, Fujikake *et al.* (2005) identified alternative isoforms of *Hsf* with two isoforms, *B* and *D* differentially elicited under heat and cold stress, respectively, suggesting that in addition to post-transcriptional modifications, transcription of the gene is autoregulated during thermal stress via alternative splicing. We examined the four isoforms *RA-D* and report three different reaction norms including increasing (*RA*), bell shaped (*RB*) and decreasing (*RC* and *RD*). This speaks of the complexity of the locus but also highlights the complex nature of transcript-level phenotypes where gene isoforms may present as separate traits. Understanding the evolution of traits ultimately depends on how traits are measured here for a gene and also for quantitative traits; for instance, the reaction norms for the final traits of insect size and growth revealed less genetic variation for plasticity than thermal performance curves for growth rate (Kingsolver *et al.*, 2004).

Conclusion

In conclusion, we found that populations from tropical and temperate east Australia exhibit similar thermal plasticity for quantitative traits despite evidence for

genetic variation for trait values. Our data therefore do not support a model of thermal evolution by plastic shifts but rather in overall trait means. We found no evidence for 'hotter is better' for performance; rather, an overall better performance of the temperate population was observed. Our study also incorporated an expanded trait set including a subset of genes exhibiting expression G × E from transcriptome studies, and whereas there was a higher degree of thermal plasticity for transcript traits, we found little support for a role of genetic variation in maintaining expression plasticity. Instead, we found most overlap in reaction norm shape for expression traits with another study with a similar thermal regime in contrast to studies using fewer exposures. This highlights the need to adequately sample thermal environments when examining the relative contribution of plasticity vs. trait mean divergence in populations. Further, the additional complexity in reaction norms between distinct isoforms of the *Hsf* gene demonstrates the importance of trait definition when inferring patterns of plastic and evolutionary responses.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Wing vein landmark points used to determine wing centroid size. For details see Materials and Methods.

Figure S2 Thermal developmental reaction norms for temperate (black) and tropical (grey) flies developed at 18 °C and 30 °C for the twelve transcripts common to this study and Levine *et al.* (2011).

Table S1 Number of generations that each population (Te = Temperate; Tr = Tropical) was maintained in the laboratory at 25 °C before each assay was performed.

Table S2 Primer sequences used for real-time PCR. *Hsf* transcript sequences taken from Fujikake *et al.* (2005).

Table S3 Bayesian Information Criterion (BIC) values for functions fitted to heat tolerance, cold resistance, egg-to-adult viability and transcript abundance data.
Table S4 Linear regression (heat resistance and body size) and multiple non-linear regression (Cold resistance and egg-to-adult viability) results for the quantitative traits.
Table S5 Three-way fixed-effects general linear-model ANOVA results for transcript abundance for the effects of developmental temperature, population (temperate vs. tropical), and the interaction between them*.
Table S6 Multiple linear and non-linear regression for transcript abundance data.
Table S7 Three-way fixed-effects general linear-model ANOVA results for transcript abundance showing the effects of developmental temperature, population, and the interaction between them for temperate and tropical flies developed at 18 °C and 30 °C for the twelve transcripts common to this study and Levine *et al.* (2011).

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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

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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

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

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.

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

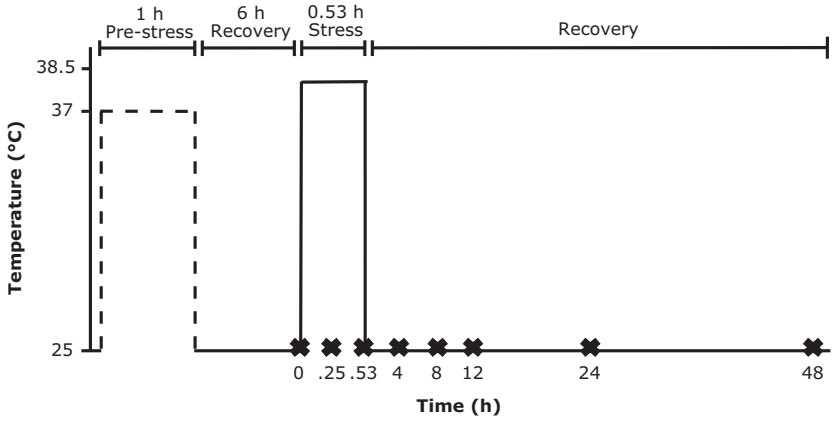

Fig. 1 Treatment and sampling schematic for the two thermal regimes, nonlethal hardening treatment (dashed box) and subsequent severe thermal stress (solid box). Prior to severe thermal stress, groups of 20 female flies either underwent 1-h exposure to 37 °C followed by 6-h recovery at 25 °C or were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

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

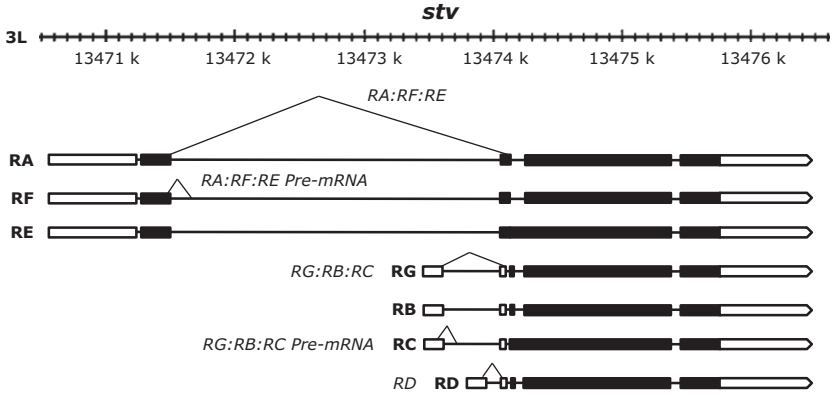

Fig. 2 *stv* gene model showing the gene region (chromosome 3L:13470641-13476615), long *RA:RE:RF* isoform subset, short isoform subset *RB:RC:RG* and smallest *RD* isoform each derived by alternative start exons. The lines joining exon junctions indicate primer sites for processed (alternatively transcribed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

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.

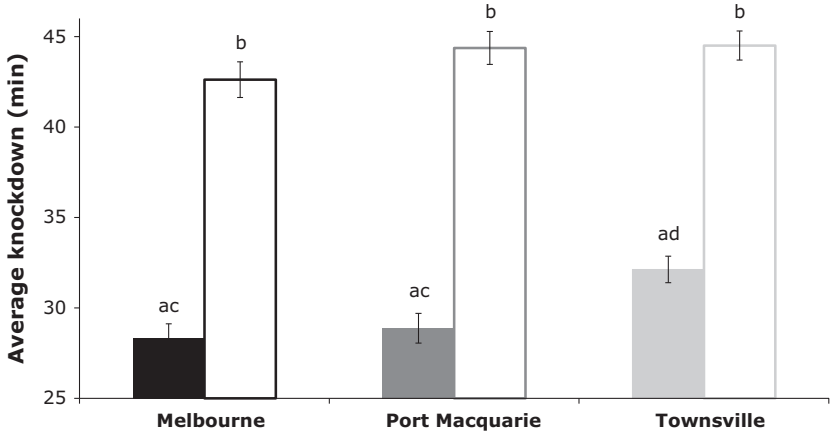

Fig. 3 Average heat knockdown of individual wild-derived *Drosophila melanogaster* females from three Australian east coast locations representing 'high' latitude (Melbourne, black fill), 'mid' latitude (Port Macquarie grey fill) and 'low' latitude (Townsville light grey fill) for basal tolerance (closed 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

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).

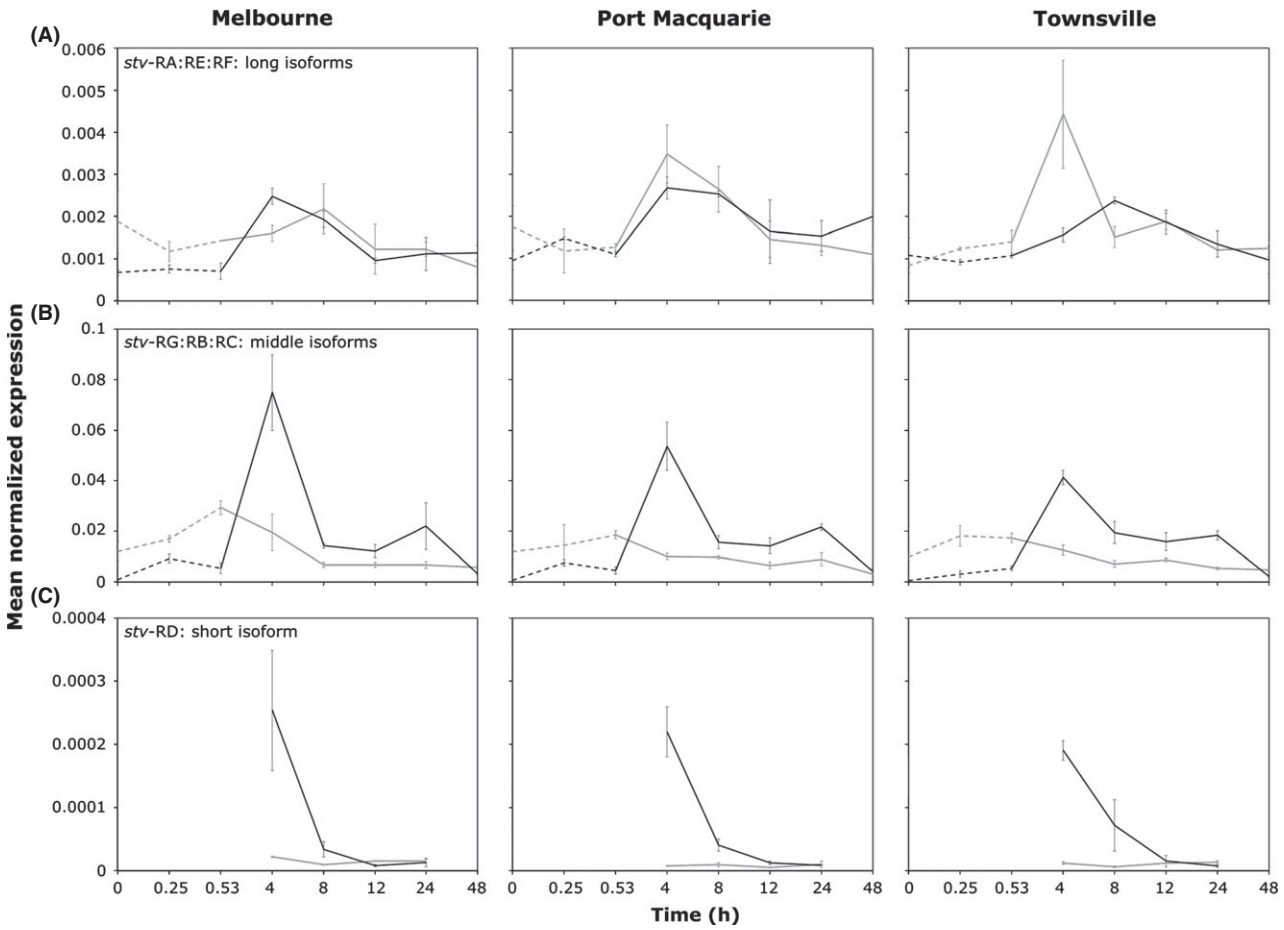

Fig. 4 Geographic, temporal and isoform variation in expression of *stv* under two different thermal regimes. All isoform/subsets are shown relative *RPL11*, dashed lines = heat shock at 38.5 °C, solid lines = recovery at 25 °C after exposure for 31.5 min. Black lines = unhardened expression, grey lines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

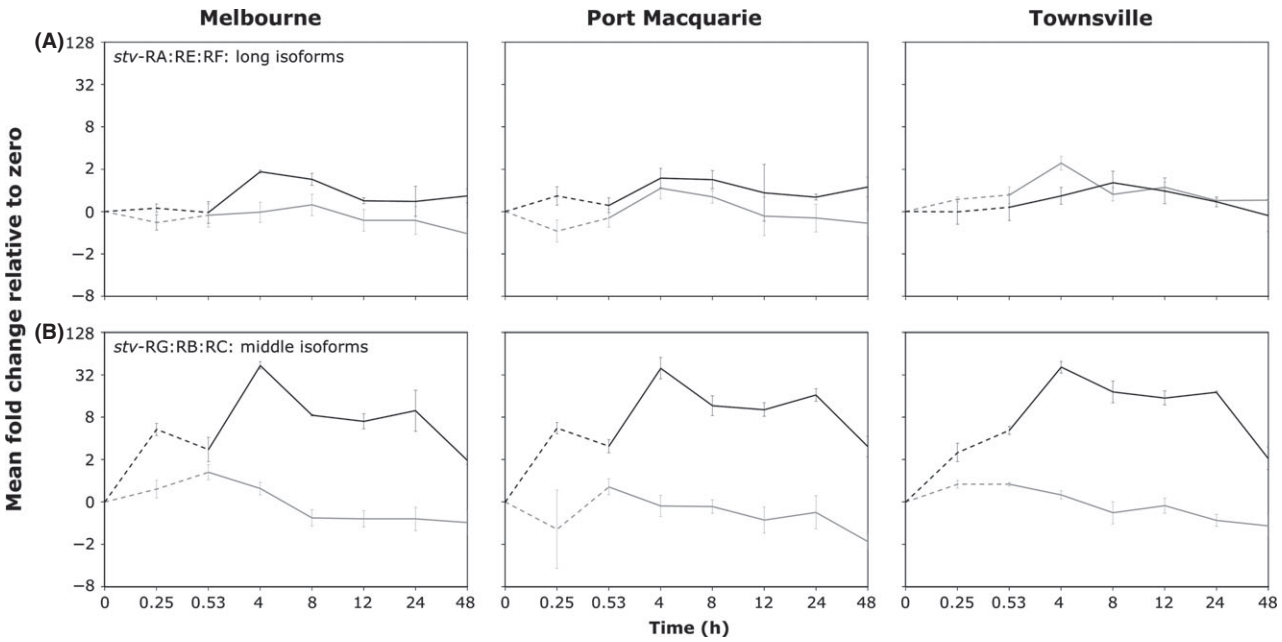

Fig. 5 *stv* isoform fold change induction relative to prestress. Dashed lines = heat shock at 38.5 °C, solid lines = recovery at 25 °C after exposure for 31.5 min. Black lines = unhardened expression, grey lines = expression following hardening at 37 °C for 1 h prestress. (A) Long isoforms (*RA:RE:RF*). 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

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-

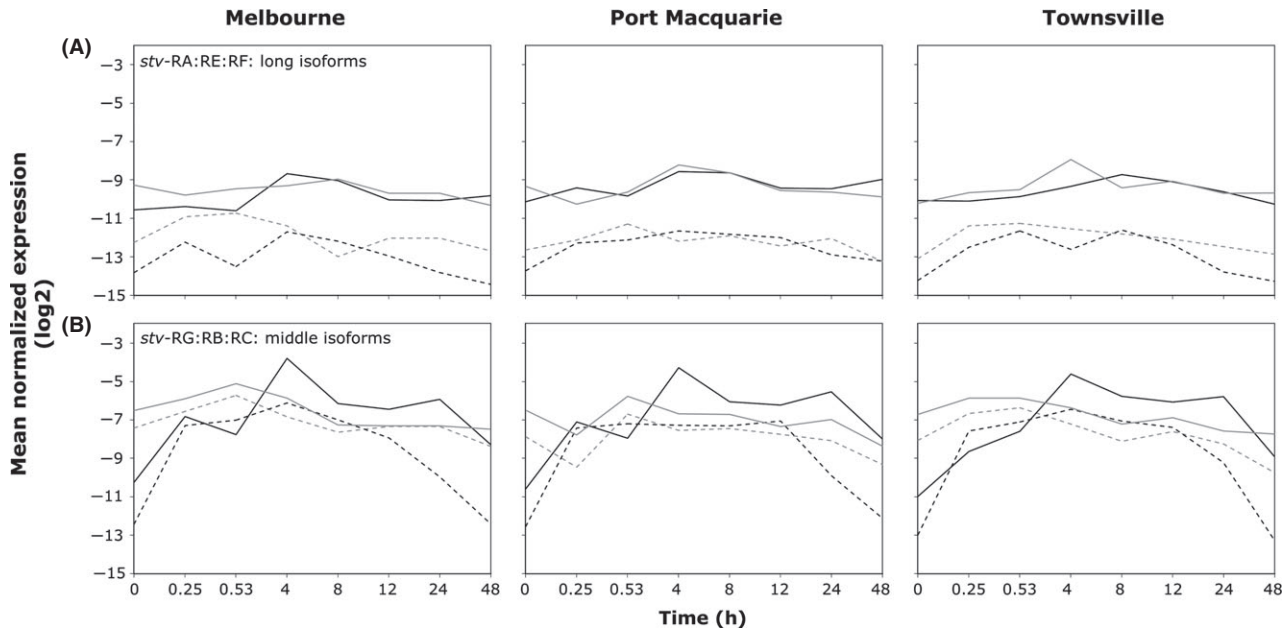

Fig. 6 *stv* pre- and mature mRNA isoform expression under two thermal regimes. Transcripts are shown log2 transformed relative to *RPL11* to directly contrast primary (dashed lines) and processed (solid lines) mRNA levels. Basal flies are shown in black, hardened are shown in grey. (A) Long isoform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

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:*

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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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.

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.