Thermal tolerance variation in natural populations of *Drosophila melanogaster*: the role of two genes and their mode of action

Submitted by

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Abstract

Since heat stress affects most organisms it is important that we understand how adaptation occurs to increasingly warm environment, especially the underlying changes in physiology, biochemistry and genetics. Few studies have shown links between physiological mechanisms and heat tolerance phenotypes in an adaptive context. Therefore the overall aim of this thesis was to use the model organism *Drosophila melanogaster* to elucidate the role of two heat-tolerance candidate genes *hsr-omega* and *hsp90* in thermal adaptation, and to look at this in a physiological context which included examining rates of protein synthesis, a postulated underlying process.

Using geographically diverse populations of *D. melanogaster* from eastern Australia I found that heat tolerance is a plastic trait that depends on rearing temperature and heat-stimulus conditions, and that the adaptive latitudinal clines in heat tolerance depend on these rearing conditions. Protein synthesis rate showed latitudinal clines that also depend on both the temperature at which flies are reared (18 or 25 °C) and heat-stress conditions (either unstressed (basal) or following a 37 °C heat stimulus), and these clines ran in parallel to clines in heat knockdown tolerance, although no evidence that the clines are connected was obtained. Consistent negative correlations between variation in protein synthesis rate and heat knockdown tolerance in a derived North/South hybrid population confirmed the importance of protein synthesis rate as a factor underlying heat tolerance variation *within* populations. However the latitudinal cline in protein synthesis did not help explain the latitudinal heat tolerance variation as this would require a positive association between the two variables. A gene thought to help control rates of general protein synthesis following heat stimulus, *hsr-omega,* was investigated for changes in expression across latitude. Clines in basal and heat-stimulated *omega-n* transcript level suggest that there is adaptive genetic differentiation in *hsr-omega* expression between populations from different climatic regions.

I show for the first time evidence for a link between expression of another heat shock gene, *hsp90*, and adult heat knockdown tolerance. Tissue levels of *hsp90* transcript and protein were negatively associated with tolerance in several independent data sets. Further, this negative association extended to a set of populations from different thermal niches and revealed a positive linear latitudinal cline for both basal *hsp90* transcript and protein level. These data suggest that heritable variation in *hsp90* expression contributes to traits that facilitate adaptation to different climatic regions, including the clinal variation in thermal tolerance. I also discuss a plausible causal role for *hsp90* as a negative regulator of the cellular heat shock response that predicts the above negative *hsp90*-tolerance association, particularly the interaction between Hsp90 protein and Heat shock factor.

Overall these data make a significant contribution to understanding the process of adaption to divergent thermal habitats and to the cellular processes and genes that facilitate thermal adaptation.

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

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 two original papers published in peer reviewed journals and one unpublished publications. The core theme of the thesis is the role of two genes and their mode of action in thermal adaptation in *Drosophila melanogaster.* The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Associate Professor Carla M. Sgrò and Associate Professor Stephen W. McKechnie.

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

In the case of Chapter 2 my contribution to the work involved the following:

The experiments were conceived and designed by Stephen McKechnie and myself. Carla Sgrò assisted with statistical analysis and manuscript input comprising 10% of the manuscript. I performed 95% of the research. I was primarily responsible for the statistical analysis and primary author of the manuscript contributing 70% with the remaining 20% written by Stephen McKechnie. This thesis chapter has been published in the Journal of Insect Physiology.

In the case of Chapter 3 my contribution to the work involved the following:

The experiments were conceived and designed by Stephen McKechnie and myself. Carla Sgrò assisted with statistical analysis and manuscript input comprising 5% of the manuscript. I performed 95% of the research. I was primarily responsible for the statistical analysis and primary author of the manuscript contributing 70% with the remaining 30% written by Stephen McKechnie. This thesis chapter has been published in the Journal of Thermal Biology.

In the case of Chapter 4 my contribution to the work involved the following:

My experiments were conceived and designed by Stephen McKechnie and myself. Travis Johnson and Louise Jensen independently conceived and designed their experiments comprising 25% and 15% respectively, of the research. Carla Sgrò assisted with statistical analysis and manuscript input comprising 15% of the manuscript. I was primarily responsible for the statistical analysis and primary author of the manuscript contributing 70% with the remaining 15% written by Stephen McKechnie. This thesis chapter has been submitted to Molecular Ecology.

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Latitudinal clines in heat tolerance, protein synthesis rate and transcript level of a candidate gene in *Drosophila melanogaster*	Published	50% Concept and design 95% Research 70% Analysis 70% Manuscript preparation
3	Capacity for protein synthesis following heat stimulus of *Drosophila* associates with heat tolerance but does not underlie the latitudinal tolerance cline	Published	50% Concept and design 95% Research 70% Analysis 70% Manuscript preparation
4	*Hsp90* levels in natural populations of *Drosophila melanogaster* cline with latitude and associate with adaptive heat tolerance variation	Submitted	50% Concept and design 60% Research 70% Analysis 70% Manuscript preparation

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis. I have also made minor corrections such as spelling errors. These papers are attached to the end of this thesis.

Additional papers not included as part of this thesis (my contribution 10%) are attached to the end of this thesis.

Signed: ……

Date: ……

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CHAPTER 1

Introduction

1.1 General

All organisms are greatly impacted by their surroundings and it is usual for a wide range of stressful environments to be encountered throughout a lifetime, including thermal extremes, low humidity and food scarcity (Andrewartha and Birch 1954; Cossins and Bowler 1987; Walther *et al.* 2002). With global warming temperature increase is fast becoming one of the biggest threats faced by species, and this is contributing to changes in species' distributions and abundances (Parmesan 2006; Raza *et al.* 2015). Therefore understanding the evolution of thermal tolerance and the biochemical and physiological mechanisms utilized by organisms to overcome the negative effects of thermal stress is increasingly important since it may enable us to enhance the survival of threatened species and help us to engineer agricultural species to maintain and improve productivity (Stinchcombe and Hoekstra 2008).

Current models of global warming predict increases in average temperature anywhere from 2 to 4 °C over the next century (Parry *et al.* 2007; Anderson 2011; IPCC 2013). Rising temperatures have already been shown to influence species distributions across a range of environments, two examples include a change in migration patterns of birds in Europe that has occurred in response to increased temperatures in the Northern Hemisphere (Sokolov 2006), and decreased survival of desert-dwelling banner-tailed kangaroo rats (Moses *et al.* 2012). Many other organisms have also been affected by increased temperatures (reviewed in Hoffmann 2010) and whole ecosystems are responding to global warming (e.g. Norris *et al.* 2013).

Ectothermic organisms are particularly susceptible to thermal stresses as they cannot internally regulate their body temperature. Almost all physiological and biochemical functions of ectotherms and many crucial behaviours are impacted by temperature, including cell membrane structure (Su *et al.* 2009), locomotion (Lachenicht *et al.* 2010), metabolism (Holzman and McManus 1973; Berrigan 1997), immune function (Le Morvan *et al.* 1998; Karl *et al.* 2011) and mating ability (Saeki *et al.* 2005; Amin *et al.* 2010). As a consequence ectotherms that are

threatened by extreme temperatures rely on genetic variations in life history traits to adapt, and/or biochemical, physiological and behavioural mechanisms to aid in survival. For ectotherms numerous studies have demonstrated a direct link between environmental temperature and stress resistance, both within and between species (Zeilstra and Fischer 2005; Overgaard *et al.* 2008; van Heerwaarden and Sgro 2010; Oliver and Palumbi 2011).

1.2 Heat tolerance

When organisms are exposed to temperatures outside of their optimal range significant changes occur to their cellular physiology and behavioural patterns. Small changes can be tolerated but large changes threaten the species. There are three consequences for a species threatened by global warming - migration, thermal adaptation or extinction. If individuals of a species cannot employ cellular mechanisms to protect their reproductive capacity, or behavioural mechanisms to escape from the heat, they will succumb to death and local populations will vanish (Cowles and Bogert 1944; Hoffmann and Parsons 1991; Hoffmann 2010). Many species will migrate to regions that have not yet been affected by the change in climatic conditions, which can often mean species distributions become more restricted (e.g. Wilson *et al.* 2007). For example nine species of small mammals in the North American Great Lakes region have shifted their range latitudinally in response to warmer climates (Myers *et al.* 2009). Range expansions have also been observed (reviewed in Chown *et al.* 2010) - one example being the expanded range of the invasive wetland plant *Lythrum salicaria* from southern to northern America (Colautti and Barrett 2013). What are the processes that enable a species to cope with thermal stress?

1.2.1 Thermal plasticity or adaptive evolutionary change

Persistence in an increasingly uninhabitable environment can involve either plastic responses or adaptive evolutionary change, and both can dramatically improve the chance of survival (reviewed in Pigliucci 2005; Hoffmann and Willi 2008; Hoffmann and Sgrò 2011). An

ectotherm's thermal environment differs markedly depending on the season, depending upon where it is situated, and/or depending upon the time of day. Some populations may occur in a warm and stable tropical environment and others in a more temperate but highly variable environment where temperatures peak at alternate extremes in the course of a single day (Hoffmann 2010). Plasticity can be extremely important for the continued survival of many species (Hoffmann and Willi 2008; Angilletta Jr 2009; Somero 2010). For example, a population of great tits from the United Kingdom has been able to solely employ phenotypic plasticity to closely track the ever changing environment in order to survive and persist (Charmantier *et al.* 2008). The ability to adjust to large daily temperature changes and the ability to seasonally acclimate are especially important traits for an ectothermic species (Bradshaw *et al.* 2004; Paaijmans *et al.* 2013). These plastic responses are often characterised in laboratory studies and have been distinguished according to the length of exposure to warmer temperatures - a brief exposure lasting a few hours induces a so-called *hardening* response, and a longer exposure for greater than about a day is referred to as an *acclimation* response (Cossins and Bowler 1987; Hoffmann *et al.* 2003). Short- or long-term changes occur to the organism's physiological response processes, and a consequential increase in general heat tolerance is the result (reviewed in Hoffmann *et al.* 2003).

The genes involved in plastic responses are still largely unknown and there is debate over whether there are specialised 'plasticity genes' or that the response is simply the modification of expression of basic genes that facilitate normal physiological processes (West-Eberhard 1989; Via *et al.* 1995; Pigliucci 2005; Telonis-Scott *et al.* 2014). Thermal plasticity certainly has a genetic underpinning – it can therefore evolve through selection. The driving force behind such evolution is thought to be thermal heterogeneity of the environment (Janzen 1967; Levins 1969; Hoffmann and Watson 1993; Ghalambor *et al.* 2006; Chown and Terblanche 2007). Organisms from the tropics experience relatively little daily or seasonal variation in thermal environment compared to temperate populations (Hoffmann 2010). This has led to the hypothesis that plasticity will be higher in temperate than in tropical populations as they have been exposed to a

greater range of environmental conditions (Tewksbury *et al.* 2008; Angilletta Jr 2009). However evidence for this is ambiguous; inter-specific studies have not found this to be the case for heat-hardening ability when tropical and other wide-spread species are compared (Mitchell *et al.* 2011; Overgaard *et al.* 2011). Intra-specific studies on the other hand have even found the opposite pattern (Sgrò *et al.* 2010). Moreover, in *D. serrata* and related species development rate has been shown to be more plastic in tropical species, whereas body size is more plastic in temperate species (Liefting *et al.* 2009). More studies of the plastic response to heat over a range of climate types are required to settle the tropical versus temperate debate on the evolution of thermal plasticity.

One benefit of heat-hardening exposure is the increased chance of survival and reproductive success from harsher temperatures that may follow. However there is evidence that detrimental fitness costs may also be involved with such hardening (Krebs and Loeschcke 1994a, 1994b; Hoffmann 1995; Bubliy *et al.* 2002), including reduced adult feeding (Huang *et al.* 2007) and reduced fecundity when conditions return to normal (Krebs and Loeschcke 1994b). However, Van Buskirk and Steiner (2009) have recently emphasised that there is little evidence for a cost to plasticity and that any cost depends upon the environment that follows the hardening or acclimation period.

Not all species have the plasticity to facilitate survival and reproduction nor can they evolve and adapt to specific environmental stress. There are specialist species that are likely to lack the genetic variation necessary to adapt to climate change - see Hoffmann *et al.* (2003), Kellermann *et al.* (2006), and Kellermann *et al.* (2009) for examples from the genus *Drosophila*. Another example is a population of intertidal copepod *Tigriopus californicus* that was selected for increased heat tolerance and reached a plateau after selection for only a few generations, suggesting a 'lack of additive genetic variation' for this trait and an inability to evolve tolerance (Kelly *et al.* 2013).

On the other hand adaptive evolution in response to climate change has now been well documented (Parmesan 2006). One example of a recently evolved adaptation includes the prokaryotic Pompeii worm that has become highly thermo-resistant (Jollivet *et al.* 2012). Also, because climate warming has caused increased periods of drought in regions of California where the mustard plant *Brassica rapa* is found, the resulting natural selection imposed on these plants has led to the evolution of early onset of flowering (Franks *et al.* 2006). A more subtle adaptive change in *Drosophila*, involved the latitudinal cline in the alcohol dehydrogenase polymorphism where a shift in allele frequency over a period of 20 years occurred, demonstrating an evolutionary genetic change linked directly to climatic warming (Umina *et al.* 2005).

1.2.2 Geographical variations in thermal tolerance

While comparative studies of related species from diverse climatic regions have detected differences in thermal tolerance (Stratman and Markow 1998; Garbuz *et al.* 2002; Kimura 2004; Kellermann *et al.* 2006; Nyamukondiwa *et al.* 2011; Strachan *et al.* 2011) a more detailed understanding of life-history and fitness traits and of the physiology and genetic changes involved with thermal adaptation is needed. Studies of variation among climatically diverse populations of a single species have higher potential to show how climatic stresses mould thermal adaptation, and provide a more detailed understanding of how adaptive evolution occurs (e.g. Guerra *et al.* 1997; Hoffmann *et al.* 2005; Rashkovetsky *et al.* 2006; Sgrò *et al.* 2010; Fallis *et al.* 2011; van Heerwaarden *et al.* 2012).

One way to study the effect of natural selection is to examine geographical clinal variation in traits, where clinal variation refers to the gradual changes in environmental variables such as temperature, rainfall, photoperiod, food resources or predators, such as along an altitudinal or latitudinal transect (cline). Clinal studies involve looking for differences in phenotype or genotype between populations taken from points along the gradients (e.g. Hoffmann and Weeks 2007; Collinge *et al.* 2008; Sgrò *et al.* 2010; Lee *et al.* 2011b; Chen *et al.* 2012; Montesinos-

Navarro *et al.* 2012; van Heerwaarden *et al.* 2012). Parallel clines in phenotype and genotype often occur in nature and allow us to look for re-occurring links to environmental variables, along similar environmental gradients on several continents for example. Consistent patterns of association between fitness traits and climatic gradients provide the primary evidence that the trait is related to adaptive genetic changes.

The cosmopolitan generalist ectotherm *D. melanogaster* is ideal for studying quantitative phenotypic variation in heat tolerance as the relevant genetics, biochemistry, physiology and life-history traits are relatively well understood, and the species is found in a wide range of environments and on nearly all continents (Hoffmann 2010). By forging a clear understanding of adaptive heat tolerance evolution in *Drosophila* we can provide a model that can be tested in other ectotherms.

1.3 Heat tolerance variation in natural *Drosophila* populations

Most natural populations of *D. melanogaster* contain significant levels of variation in heat tolerance, and this variation has a genetic basis both within and between populations (Krebs and Feder 1997b; Hoffmann *et al.* 2002; Sgrò *et al.* 2010; Fallis *et al.* 2011). The most thoroughly studied clinal heat-tolerance patterns are those of *D. melanogaster* along the east coast of Australia (Hoffmann and Weeks 2007). Adult heat-knockdown tolerance varies with latitude with tropical northern populations having higher heat tolerance than temperate southern populations (Hoffmann *et al.* 2002; Sgrò *et al.* 2010). Tolerance to heat can be measured in different ways, some of which are likely to be more ecologically relevant than others (Terblanche *et al.* 2011). Whereas adult heat knockdown time, measured either before or after a period of rapid heat acclimation, is used to demonstrate adaptive geographical variation (Sgrò *et al.* 2010) other heat-related fitness components, such as reproductive success following heat stress, may be more significant from an ecological and evolutionary perspective. While not all measures of heat tolerance variation are correlated (Hoffmann *et al.* 1997) many underlying

cellular components that influence different measures of heat tolerance are likely to be shared in common. Elucidating the genetic and cellular basis of adult heat-knockdown heat tolerance variation along a thermal environmental gradient in *Drosophila* provides a promising opportunity to begin to understand the differential thermal adaptation across climatic regions.

Many other traits in *D. melanogaster,* traits not directly related to surviving extremes of heat stress, also vary along latitudinal gradients, including morphological traits such as wing and body size (James *et al.* 1995), life history traits including development and egg production timing (James and Partridge 1995), and cold tolerance, with more tolerant populations occurring at cool-temperate latitudes (Hoffmann *et al.* 2002). Numerous genetic markers, such as chromosomal inversions (Hoffmann and Weeks 2007), allozyme loci such as *Adh* and *α-Gpdh* (Oakeshott *et al.* 1982), and the myriads of genes now identified by genomic studies as being climatically differentiated either by genotype or expression patterns (Turner *et al.* 2008; Kolaczkowski *et al.* 2011; Levine *et al.* 2011) also vary along latitudinal gradients and are likely to underlie clinal trait variation. The issue becomes one of relating the adaptive trait variations, in this case heat tolerance, to the clinally differentiated genes in a meaningful causal context. A way forward for a specific adaptive trait is to identify the relevant intermediate physiology or cell biochemistry and to link this to gene products and genes that facilitate the changed cellular processes involved (Stinchcombe and Hoekstra 2008; Blackman 2010; Nadeau and Jiggins 2010). Some recent studies have aimed at dissecting specific genes that underlie traits involved with climatic adaptation in general (e.g. Levine and Begun 2008; McKechnie *et al.* 2010; Paaby *et al.* 2010). One useful approach is to study the expression patterns of candidate genes and proteins across an environmental gradient, allowing quantitative trait variations to be more readily linked to specific genetic variation (Whitehead and Crawford 2006; Swindell *et al.* 2007; Lee *et al.* 2011b).

1.4 Cellular processes involved when *Drosophila* responds to heat stress

When organisms are exposed to temperatures significantly above their optimal range, and to other environmental stresses, there is a change to normal cellular processes known as the cellular stress response (Kültz 2005). This highly conserved response involves many hundreds of cell-stress response genes and proteins that have been discovered by contemporary genomic and proteomic studies over the last 15 years. The function of many of these genes are just beginning to be understood and they include effects on respiratory metabolism, redox regulation, lipid and energy metabolism and protein degradation mechanisms, to name a few (Kültz 2005). In *Drosophila* the genomics of the heat stress response in particular has been well characterised and involves changes to regulation of many hundreds of genes (Leemans *et al.* 2000; Sørensen *et al.* 2005; Laayouni *et al.* 2007; Telonis-Scott *et al.* 2013). Key insights into adaptive heat tolerance in *Drosophila* can be gleaned from one particular genomic study that examined changes to gene regulation in a population selected to be heat tolerant (Nielsen *et al.* 2006). Of 118 genes expressed constitutively at higher or lower levels in this population, 102 were the same as those that were up- or down-regulated in response to an abrupt heat shock to an intolerant population (and regulated in the same direction). Again, many cellular processes were implicated in the heat tolerant population, including changes to signal transduction, changes in transcription, proteolysis, carbohydrate and fatty acid metabolism, but particularly to genes involved with photo-transduction (Nielsen *et al.* 2006). Also helpful, is the identification of changes to gene expression, and implicated cellular processes, in *Drosophila* populations cultured at 30 °C compared to culture at 18 °C, and in two different populations - one derived from a cool temperate climate and one from a tropical climate (Levine *et al.* 2011). Similar cellular processes to those suggested by the former *Drosophila* genomic studies were implicated. However, despite these insights few of the cellular processes revealed have led to any in-depth understanding of physiological mechanisms that underlie natural geographic variation in adult heat knockdown tolerance. As a way forward, to establish new links between such adaptive heat tolerance variation and genetic variations, I focus here on the classic heat shock response, a well-

understood and primary sub-component of the cellular stress response. The heat shock response has been extensively investigated over the last 50 years (Pardue *et al.* 1992; Feder and Hofmann 1999).

The heat shock response was first detected at the chromosomal level by Ritossa (1962) in *Drosophila bucksii.* This component is a highly conserved and is vital for heat survival not only in *Drosophila,* but in every organism in which it has been studied (Parsell and Lindquist 1993). It involves up-regulation of the heat shock protein genes (*hsps*) that protect the cell from damage that would otherwise be caused by the stressful high temperatures. During heat shock, puffing is observed in specific regions of the *Drosophila* polytene chromosomes (Ritossa 1962). These puffed regions indicate sites of highly active transcription and five of the six major puff regions correspond to the genes encoding these heat shock proteins (Hsps). This up-regulation of *hsp* synthesis is accompanied by reduced synthesis of general heat-sensitive proteins that are required for housekeeping cellular functions (Lindquist 1980). Shutting down production of normal proteins that would be denatured by heat is clearly adaptive, if not only because it conserves energy otherwise wasted on synthesis of a product that is rendered useless by heat denaturation. The one puff that does not harbour an *hsp* gene, at cytological position 93D in *D. melanogaster,* is the site of up-regulation of the heat shock RNA-producing gene *hsr-omega* (Pardue *et al.* 1990). This gene is involved with the repression and/or modulation of general protein synthesis following heat stress (Lakhotia 2003; Johnson *et al.* 2011).

1.4.1 Initiation of the heat shock response via Heat shock factor

The heat shock response is primarily regulated at the level of transcription (Schöffl *et al.* 1998). The genes are regulated by the binding of heat shock transcription factor (Hsf) to specific DNA sequences in the promoters of the heat shock response genes (Amin *et al.* 1988; Baler *et al.* 1993; Fernandes *et al.* 1994). These sequences are known as heat shock elements (HSEs) and they consist of three short inverted repeats of n(GAA)n found in the regulatory regions of genes

regulated by heat stress, particularly all heat shock proteins (Amin *et al.* 1988) and *hsr-omega* (Garbe *et al.* 1989). Under normal conditions Hsf exists as a small inactive protein monomer that abides in the cell in complex with molecules such as p23, PP5, Hsp70, Hip, Hop and Hsp90 (Bharadwaj *et al.* 1999). In *Drosophila* the *hsp90* gene is referred to as *hsp83* and the protein as Hsp83 - for consistency throughout this thesis the *Drosophila* Hsp83 gene will be referred to as *hsp90* and its protein as Hsp90. Upon heat shock Hsf dissociates from these complexes, translocates to the nucleus where phosphorylation and trimerisation occurs, leading to its binding to the HSEs (Wu 1995). As a result the *hsp* genes are up-regulated, along with regulation of many other genes (Birch-Machin *et al.* 2005). *Hsf* is highly conserved in the animal kingdom and most vertebrates have multiple copies of the gene, whereas many invertebrates have only one copy (reviewed by Wu 1995). While mammals have four *hsf* genes, only *hsf1* responds to heat in a similar way to invertebrate *hsf* (Baler *et al.* 1993; Sarge *et al.* 1993).

1.4.2 Heat shock proteins

The Hsps are heat-inducible proteins, found in a range of organisms and are highly evolutionarily conserved (Feder and Hofmann 1999; Sørensen *et al.* 2003). Hsps are molecular chaperones that bind to many house-keeping proteins helping to prevent denaturation, aid protein folding and refolding, and prevent partially denatured proteins from forming aggregates (Somero 1995). Aggregated proteins are harmful to the cell; they can cause disease, lead to cell death, and can be fatal to the organism (Houck *et al.* 2012). There are many different *hsps*, named and classified into families according to their molecular weight. The main *Drosophila* inducible *hsps* include *hsp90*, *hsp70*, *hsp68*, *hsp40* and the cytologically clustered small *hsps*; *hsp22*, *23*, *26* and *27* (reviewed in Zhao and Jones 2012). Each *hsp* performs different, yet often complementary functions, and are expressed differentially during development and in different tissues. They work in complex with other proteins to prevent denatured proteins from harming the cell (reviewed in Tutar and Tutar 2010).

Some *hsps* are expressed under non-stress conditions, as well as being heat-regulated, as they are essential for normal cell function, performing roles such as controlling protein conformational changes, regulating the function of other proteins, regulating protein degradation, promoting translocation of proteins across cellular membranes and aiding in protein folding (Tutar and Tutar 2010). These constitutively expressed *hsps* include *hsp90* and heat shock cognates such as *hsc70* and *hscs1-7* (Craig *et al.* 1983; Lindquist and Craig 1988). Hsps are also under investigation for a role in prevention of diseases characterised by misfolded, aggregated proteins (Kaul and Thippeswamy 2011; Kargul and Laurent 2012).

The predominant heat shock protein following heat stress is Hsp70. Hsp70 is produced at negligible levels, if at all, in cells at normal temperatures and is up-regulated 1000 fold upon heat stress (Velazquez *et al.* 1983; Lindquist 1986). The main role of Hsp70 is to protect cells against heat by binding and chaperoning substrate proteins, folding of substrate proteins, helping to translocate target proteins across membranes and targeting proteins for degradation (Mayer and Bukau 2005). Fast production of Hsp70 in ectotherms following a heat stress appears essential since numerous molecular mechanisms have evolved to expedite its production. These include, *a)* the absence of introns that require time-consuming transcript processing, as is the case for the other inducible *hsp* genes (Yost and Lindquist 1986), *b)* multiple copies of the *hsp70* gene in the genome facilitate higher rates of Hsp70 protein accumulation (Gong and Golic 2006), *c)* fast initiation of transcription occurs facilitated by RNA polymerase II already in place, 'stalled', on the *hsp70* promoter (Rasmussen and Lis 1993), and *d) hsp70 mRNA* has little minimal 5' UTR secondary structure that expedites fast translation (Hess and Duncan 1996). When heat stress subsides the synthesis of Hsp70 is repressed (DiDomenico *et al.* 1982a), suggesting that it is detrimental to the cell under non-stress conditions. High concentrations of Hsps can be toxic to the cell (Krebs and Feder 1997a). The negative consequences of hardening or acclimation on some fitness traits could be due to the energetic strain of producing large quantities of the Hsps (Krebs and Feder 1998).

While the heat shock response is highly conserved it has been fine-tuned by natural selection according to the particular range of temperature exposures for the species in question. For example, lower-intertidal species of marine snails from climatically temperate regions have lower induction temperatures of *hsps* than more tropical, higher-intertidal species of snails (Tomanek and Somero 1999). Species that are cold-adapted have lower threshold temperatures to trigger the response, compared to related hot-adapted species (Feder and Hofmann 1999; Lerman and Feder 2001).

1.5 The heat stress genes and thermotolerance variation in *Drosophila*

In order to understand the ecological and evolutionary significance of the heat shock response and its components, it is important to consider the evidence that natural variation in these components contribute to natural heat tolerance variation. *Hsp70* has been scrutinised for its role in heat tolerance (Parsell and Lindquist 1993) and has been a major focus in efforts to relate thermotolerance variation to protective gene function (Feder and Hofmann 1999). *Drosophila* larvae appear to be protected from heat stress by Hsp70. For example, larvae inhabiting necrotic fruit that survive the extreme temperatures imposed by direct sunlight produce high amounts of Hsp70 protein (Feder *et al.* 1997). Also, in lines of *D. melanogaster* established from a natural population that experiences heat stress on a typical day, heat tolerance of first instar larvae was positively correlated with Hsp70 protein level (Krebs and Feder 1997b). However, while strains of *D. melanogaster* engineered to contain 12 extra copies of the *hsp70* gene have higher levels of *hsp70* and result in a higher adult and larval heat tolerance after hardening (Feder *et al.* 1996; Gong and Golic 2006), levels of Hsp70 in natural populations of adult *D. melanogaster* do not relate to natural heat tolerance variation (Jensen *et al.* 2010). In the latter and several other studies abrupt exposure to heat is used to assess tolerance and this causes fast knockdown of adult *Drosophila* before significant amounts of Hsp70 or other Hsps could be synthesised, offering a possible explanation for the lack of associations between *hsp* levels and knockdown time. Consistent with this idea is the fact that *hsp70* level has been associated with levels of

hardened heat tolerance (Feder *et al.* 1996) when the time-frame between initial exposure and knockdown is extended.

The *hsf* gene is a heat shock response gene that is another candidate for effects on heat tolerance (Lerman and Feder 2001). Hsf interacts quickly with at least 188 genes in *Drosophila* following heat stress, binding to the promoters of genes encoding *hsps* and other chaperones, metabolic enzymes and transcriptional regulators (Westwood *et al.* 1991; Birch-Machin *et al.* 2005). However work with a heat-sensitive *hsf* mutant strain has provided ambiguous implications for any effect it might have on heat-knockdown resistance (Nielsen *et al.* 2005; Sørensen *et al.* 2009). Little evidence exists for the influence of other Hsps on natural variation on *Drosophila* heat tolerance except for a few indirect and direct association studies (e.g. McColl *et al.* 1996; Frydenberg *et al.* 2003). In fact, one line of investigation suggests that activation of Hsf and therefore up-regulation of *hsps* is not important for high temperature heat-knockdown resistance (Nielsen *et al.* 2005).

The measurement of natural latitudinal variation in *Drosophila* heat knockdown tolerance involves abrupt exposure to a high temperature and does not allow a lot of time for new genes to be switched on or new 'protective' proteins to be synthesised. In such assessments the cells may rely on transcripts and proteins that are already present. Two genes that encode major components of the classic heat shock response are constitutively expressed in the absence of stress and could therefore influence tolerance levels in such tests - the *hsr-omega* gene and the *hsp90* gene. *Hsr-omega* is a strong candidate since it shows genotypic latitudinal clines that parallel knockdown tolerance (Anderson *et al.* 2003; Collinge *et al.* 2008), and its transcript levels changed markedly in populations selected to be heat tolerant (McKechnie *et al.* 1998). Hsp90 is a strong candidate since it has an established function to buffer Hsf levels (Duina *et al.* 1998; Zou *et al.* 1998) and therefore influence initiation of the heat shock response. Both *hsr-omega* and *hsp90* are worthy of further investigation.

1.6 Two new candidate genes for *Drosophila* natural heat tolerance variation

1.6.1 Hsr-omega *variation is related to thermotolerance variation*

The *hsr*-omega gene of *Drosophila* occurs at the site of the largest heat shock puff which is also the earliest to arise upon heat stress (Pardue *et al.* 1990). The gene is intriguing as it produces no known protein product (Fini *et al.* 1989). Similarly to the *hsps* it is constitutively expressed in almost all cells and is up-regulated in response to both temperature and chemical stresses (Bendena *et al.* 1989). *Hsr-omega* produces two main transcripts; a long nuclear-located transcript (*omega-n* transcript) and a smaller cytoplasmic-located transcript (*omega-c* transcript). These transcripts arise as a result of alternate termination of transcription of the gene (Fini *et al.* 1989). The *omega-n* transcript is unspliced, contains both exons (~600bp) and an intron (700bp) and the 3' variable length tandem repeat region (Pardue *et al.* 1990). The repeated region comprises repeat units of a 280bp sequence, the number of which vary between strains, making this region anywhere from 5 to 25 kb in length, thus *omega-n* is the largest of the transcripts with a total length of up to ~27kb (Pardue *et al.* 1990). The cytoplasmic transcript, *omega-c*, has a short open reading frame, resembling a short *mRNA* and is known to localise to ribosomes where proteins are synthetised (Pardue *et al.* 1990).

Two naturally occurring polymorphic sites occur in the *D. melanogaster hsr-omega* gene; the *hsr-omega*$^{L/S}$ indel polymorphism found in the first exon (and therefore part of both *omega-n* and *omega-c* transcripts) and the variable length tandem repeat region at the 3' end of the gene and found only in the longer nuclear *omega-n* transcript (Hogan *et al.* 1995; McKechnie *et al.* 1998). Variants at both sites show associations with thermal habitat. Firstly, a robust association occurs between latitude and the *hsr-omega*$^{L/S}$ genotypes, with higher frequency of *hsr-omega*^{S} allele occurring at tropical low latitudes in eastern Australia (McColl and McKechnie 1999; Anderson *et al.* 2003). Secondly and independently of this *L/S* variation, alleles with a lower number of

repeats in the *omega-n* transcript occur at higher frequency in tropical eastern Australian populations (Collinge *et al.* 2008). These clinal patterns correspond to clinal patterns in heat tolerance (Hoffmann *et al.* 2002; Sgrò *et al.* 2010), where low latitude populations have higher heat tolerance. In addition to these geographic-genotypic associations with heat tolerance the *hsr-omega*^{S} allele increased in frequency after 11 generations of selection for heat resistance following a hardening treatment, but not in the control lines (McColl *et al.* 1996). Indeed, the best evidence that expression of this gene influences heat tolerance, particularly adult hardened heat tolerance, comes from these heat selected lines where lines selected for high *hardened* heat tolerance evolved highly divergent constitutive levels of both transcripts compared to heat sensitive lines (McKechnie *et al.* 1998).

A key cellular function of the *omega-n* transcript is to regulate the heterogeneous ribonuclear proteins (hnRNPs) that are part of the machinery involved in splicing introns from pre-mRNAs (Krecic and Swanson 1999; Lakhotia *et al.* 1999). Almost all of the hnRNPs, normally active at many different sites on the chromosomes, re-locate to the 93D *hsr-omega* locus upon heat shock (Lakhotia *et al.* 1999). Sequestering these factors prevents splicing of pre-mRNAs that contain introns, a feature present in most housekeeping protein genes but not present in most *hsps*. Such sequestering occurs following heat shock when *omega-n* levels are up-regulated, and this is thought to be largely responsible for down-regulating synthesis of housekeeping proteins (see Fig. 1.1; Lakhotia *et al.* 2001; Johnson *et al.* 2009a). *D. melanogaster* lines genetically altered to produce little or no *omega-n* show an increase of 1.5 fold for rate of protein synthesis (Johnson *et al.* 2011).

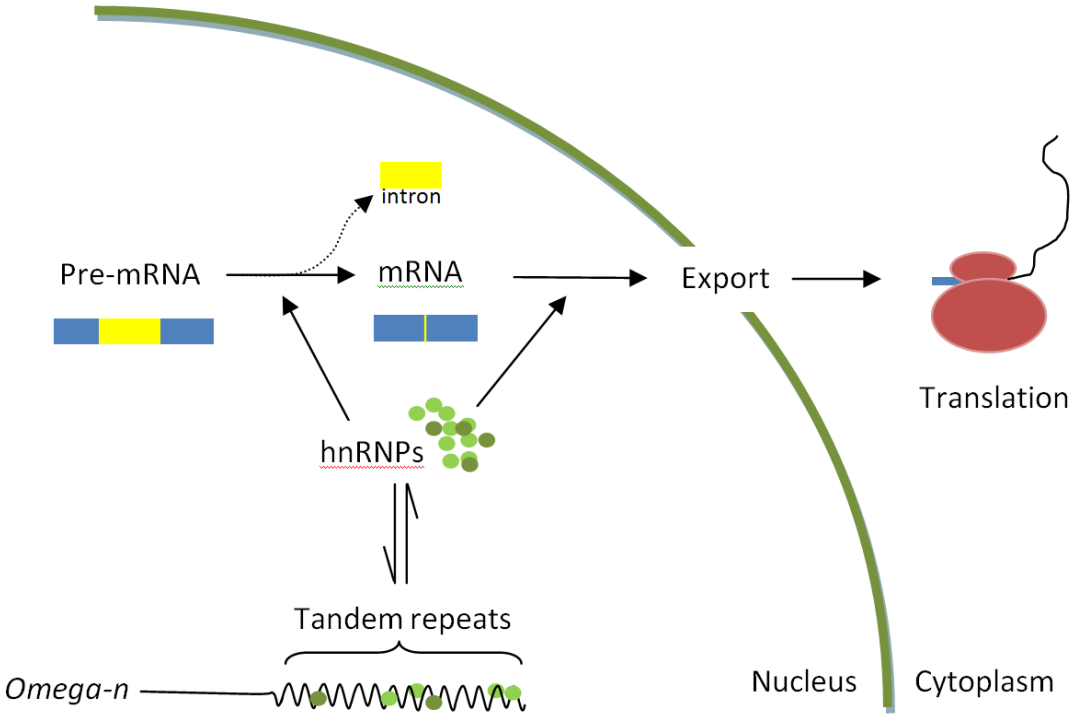


Figure 1.1 Model of *omega-n* function in the nucleus. High *omega-n* results in more sequestration of hnRNP splicing factors, removing them from the splicing machinery. Consequently a reduction in rates of mRNA maturation and export and hence lower protein synthesis rates (Johnson *et al.* 2009a).

1.6.2 Protein synthesis rate as a candidate physiological mechanism underlying heat tolerance variation

Our growing understanding of the role of *hsr-omega* in the cellular heat stress response, involving shutting down synthesis of general 'housekeeping' proteins that are denatured by heat may provide a clue to a mechanism by which *hsr-omega* influences thermotolerance. We might expect this gene to have an effect on total rates of protein synthesis following heat stress. Since the modulation of protein synthesis following heat stress is a major component of the heat stress response it has the potential to change tolerance levels. This idea is supported by genomic studies on *Drosophila's* response to heat (Leemans *et al.* 2000; Nielsen *et al.* 2006; Laayouni *et al.* 2007) that have implicated protein synthetic processes by grouping of many responsive candidate genes into the *translation* and *regulation of transcription* Gene Ontology (GO) categories.

Total rate of protein synthesis has recently been negatively related to hardening capacity (the capacity to increase heat tolerance above the basal level) in isofemale lines of *D. melanogaster* (Johnson *et al.* 2009b) - strains that synthesised proteins at a lower rate after heat stimulus had higher increases in heat knockdown time after hardening. Although this protein synthesis rate is a compound measure, involving increased synthesis of Hsps and decreased synthesis of housekeeping proteins, Johnson *et al.* (2009b) speculated that low housekeeping protein synthesis after heat shock (resulting in less denatured cellular debris) was a possible explanation for the increased hardening capacity.

Thus, control of the rate of protein synthesis following heat stress may be a mechanism utilised by *Drosophila* to affect changes in thermotolerance in response to climatic selection – an idea that warrants further investigation. While numerous mechanisms that connect heat-stimulated protein synthesis with heat tolerance are possible, the simplest idea is that faster synthesis of Hsps quickly protect from cell damage and increase heat knockdown tolerance. The intensity of the heat stress and the exact measure of heat tolerance assessed would of course be highly

relevant. While (Johnson *et al.* 2009b) have examined the relationship between total protein synthesis rate and thermal tolerance at the intra-population level, the inter-population and latitudinal relationship between these variables has not been examined.

1.6.3 Hsp90 protein

Hsp90 is abundant in all cell types (1-2% of all proteins) in the absence of stress and in thermally benign habitats, and is involved with maintaining and buffering many cell-signalling and other pathways under varying cellular conditions (Richter and Buchner 2001; Tsaytler *et al.* 2009). The usual role of Hsp90 is to interact with and stabilise a large set (> 200) of client proteins that are components of these pathways (Chiosis *et al.* 2013). The role that Hsp90 plays in the heat stress response and thermal tolerance is likely to be different to that of the other heat shock proteins (Fernandes *et al.* 1995; Shopland and Lis 1996). While it is moderately up-regulated following heat stress this occurs in a manner somewhat independent of the *hsps*, at least partly because the *hsp90* gene (unlike the other *hsps*) contains an intron, and therefore it does not 'skip' the splicing step that happens for the other non-intron-containing *hsps* (Yost and Lindquist 1986). Also, *hsp90 mRNA* has a more extensive secondary structure in the 5'-untranslated region that is likely to slow down its rate of translation (Ahmed and Duncan 2004). Hsp90 is clearly important to cells following heat shock as it interacts with the key trigger for the cellular heat shock response, Hsf (Zou *et al.* 1998), and this is conserved across many organisms, including yeast, insects, amphibians, mammals and plants (Ali *et al.* 1998; Duina *et al.* 1998; Morimoto 1998; Zou *et al.* 1998; Bharadwaj *et al.* 1999; Yamada *et al.* 2007; Shamovsky and Nudler 2008). Thus, under non-stress conditions Hsp90 has a role in regulating heat shock factor. Upon heat stress Hsp90 dissociates somewhat from Hsf, freeing Hsf monomers that trimerise under heat stress enabling the trimers to bind to heat shock elements promoting transcription of the stress genes (Zou *et al.* 1998). It follows that basal levels of Hsp90 protein may influence the availability of Hsf monomers that trigger the cellular heat shock response. Higher levels of Hsp90 might be expected to delay the appearance of active Hsf leading to a slower heat shock

response, and perhaps reduced heat tolerance. However there is currently little empirical evidence for any relationship between Hsp90 level and heat tolerance.

1.6.4 Hsp90 protein involvement in heat tolerance variation

Hsp90 has been studied extensively for a possible role in canalisation, such as the buffering of various developmental traits, bristle number and size, and even *Arabidopsis* life-history traits (e.g. Debat *et al.* 2006; Milton *et al.* 2006; reviewed in Rutherford *et al.* 2007; Sangster *et al.* 2008; Chen and Wagner 2012). Also, being a chaperone molecule, Hsp90 has been studied for its role in cellular protection (Weich *et al.* 1992; reviewed in Vaughan *et al.* 2010). Hsp90 is modestly up-regulated upon heat stress (Richter and Buchner 2001; Chiosis *et al.* 2013). It is certainly a good candidate to affect adaptive variation in adult heat knockdown tolerance (see Csermely *et al.* 1998; Sawarkar *et al.* 2012). Hsp90 however has not been characterised thoroughly in relation to heat tolerance variation in general. Seasonal and heat- and cold-stress induced changes in *hsp90* transcript level have been found in the onion maggot, *Delia antique* (Chen *et al.* 2005), and *Arabidopsis* plants treated with a *hsp90* inhibitor survived temperatures lethal to control plants, suggesting a negative association between *hsp90* and heat tolerance (McLellan *et al.* 2007). No association was found in *Drosophila* between a rare, naturally occurring, *hsp90* allele and heat resistance (Rako *et al.* 2007). On the other hand Chen and Wagner (2012) found in *Drosophila* that naturally occurring mutations in this gene reduced *hsp90* expression, reducing fitness components such as larval competition, fecundity and longevity, and also reduced heat resistance suggesting a positive association. In *Drosophila* *hsp90* is induced by heat in larvae and pupae but there was no correlation between amount of *hsp90* and heat tolerance ability. One *Drosophila* mutant with less *hsp90* was found to be less heat resistant (Chen and Wagner 2012), however since Hsp90 protein is required for many cellular processes, it is unclear whether the mutation made the strain generally unfit rather than specifically heat sensitive. These studies used single strains or populations and have found inconsistent associations between *hsp90* and thermal tolerance, and they have not examine the

gene or its protein for variation across climatic gradients - further work is needed in *Drosophila* to clarify whether or not natural variation in expression of *hsp90* has any effect on thermotolerance.

1.7 This study

In this thesis I pursue two lines of investigation about cellular aspects of the heat shock response in *Drosophila* and about how these relate to natural variation in heat tolerance. One focus is on protein synthesis before and after heat shock to see if variation in this relates to natural thermal tolerance variation. This includes further investigating the role of the *hsr-omega* gene in protein regulation and heat tolerance. The other is to investigate whether there is natural variation in expression of the *hsp90* transcript or protein and whether this relates to natural heat tolerance variation. I want to know whether or not these heat stress response genes, and mechanisms they influence, help us understand heritable variation in heat tolerance and the genetic basis of climatic adaptation.

An isofemale-line approach has proven to be an excellent tool in dissecting adaptive trait variation in many studies (e.g. Hoffmann and Parsons 1988; Weeks *et al.* 2002; McKechnie *et al.* 2010) and is the basic approach taken in part of this thesis, along with clinal study of genes and traits. Sets of lines recently established from field populations are genetically diverse and allow associations between genes, proteins and phenotypic data to be explored, while controlling for background variation when natural levels of linkage disequilibrium are present. These studies in themselves do not prove that the genes of focus directly influence the trait in question. However, when associations are under-pinned by an understanding of the cellular function, and an obvious physiological or biochemical mechanism can be linked to the trait, such studies provide excellent primary evidence of a cause and effect that deserves further manipulative investigation.

In the first result chapter (Ch 2) the possibility of a climatic gradient in rates of protein synthesis is explored. I rear clinal populations of *D. melanogaster* at either 18 or 25 °C to approximate

winter and summer growth conditions respectively, to see if any patterns found hold up under different development temperatures. I characterise these populations for heat tolerance and investigate whether rates of protein synthesis are linked to clinal variation in heat resistance, with measurements of protein synthesis rates made both before and after heat stimulus. Since the strong candidate gene for heat tolerance variation, *hsr-omega,* has recently been demonstrated to influence protein synthesis I also characterise levels of the major transcript of *hsr-omega*, *omega-n* in these same clinal populations. This work has been published in the Journal of Insect Physiology.

In order to clarify two different, apparently opposing, associations of heat tolerance with rates of protein synthesis, one involving genetic variation from a latitudinally central population and one involving multiple populations along the latitudinal cline, I assess in chapter 3 both heat tolerance and protein synthesis rate in a synthetic hybrid population generated by crossing populations of *D. melanogaster* from two latitudinally extreme locations. A negative relationship between total protein synthesis rate and heat tolerance among these hybrid lines suggests that protein synthesis rate is important for heat tolerance but is not a factor contributing to the cline in heat tolerance. This work has been published in the Journal of Thermal Biology.

In the final research chapter (Ch 4) I explore the role of the *hsp90* gene in heat tolerance variation since this strong candidate protein that has been significantly overlooked in earlier heat tolerance investigations. I test for an association between variation in levels of both the *hsp90* transcript and protein level with heat tolerance, and together with colleagues we find negative associations in four independent sets of lines and populations. I further examine its role in heat tolerance by quantifying *hsp90* gene transcript and protein level in a set of populations collected from across the latitudinal cline. This is the first study to show that the latitudinal patterns of heat tolerance are 'paralleled' by a cline in levels of *hsp90* transcript. Given the numerous cell-signalling client proteins and downstream traits influenced by Hsp90, my data suggest that

control of expression of *hsp90* may be more important in climatic adaptation than previously thought.

Monash University

Declaration for Thesis Chapter 2

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	Extent of contribution (%)
Design, research, analysis, manuscript preparation	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	Extent of contribution (%) for student co-authors only
Carla Sgrò	Analysis, manuscript preparation	10%
Stephen McKechnie	Design, analysis, manuscript preparation	20%

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**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

CHAPTER 2

Latitudinal clines in heat tolerance, protein synthesis rate and transcript level of a candidate gene in *Drosophila melanogaster*

2.1 Abstract

The occurrence of climatic adaptation in *Drosophila melanogaster* is highlighted by the presence of latitudinal clines in several quantitative traits, particularly clines in adult heat knockdown tolerance that is higher in tropical populations. However the presence of latitudinal patterns in physiological characteristics that may underlie these traits have rarely been assessed. Protein synthesis has been implicated as an important physiological process that influences thermal tolerance, and this has not been examined in a clinal context. Here, we characterise latitudinal variation in *D. melanogaster* from eastern Australia in both adult heat knockdown tolerance and rates of protein synthesis following rearing at both 25 °C, approximating summer conditions, and 18 °C approximating winter development. We also examined clinal variation in the predominant nuclear transcript of the heat-inducible RNA gene *hsr-omega*, which has been implicated in regulating protein synthesis. We find significant clines in heat-hardened tolerance when cultured at both 18 and 25 °C - tolerance increased towards the low latitude tropics. Rates of protein synthesis measured in ovarian tissue also associated negatively with latitude, however the presence of the clines depended on rearing temperature and heat stress conditions. Finally, *omega-n* levels measured without heat stress showed a positive linear cline. When measured after a mild heat stress higher levels of *omega-n* were detected and the clinal pattern became parabolic - mid-latitude populations had lower levels of the transcript. While congruent latitudinal trends were detected for these three traits, only a low level of positive association was detected between protein synthesis and thermal tolerance providing little evidence that these traits are related at the level of cellular physiology. However the new clinal patterns of protein synthesis and *hsr-omega* variation suggest that these variables exert important influences on traits involved with latitudinal climatic adaptation.

2.2 Introduction

The threat that global warming poses to the continuing existence of many species has heightened our awareness of the importance of adaptation to thermal extremes in plant and animal populations (Parmesan 1996; Deutsch *et al.* 2008). Some of the best examples of such thermal adaptation are in single species that are spread latitudinally over a broad range of climatic regions (e.g. Zani *et al.* 2005; Bahrndorff *et al.* 2006; Kuo and Sanford 2009). In one species, *Drosophila melanogaster*, regional differentiation in adult heat knockdown tolerance is well characterised (Guerra *et al.* 1997; Fallis *et al.* 2011), in particular the latitudinal cline along about 3000 km of the east Australian coast (Sgrò *et al.* 2010). The presence of clinal variation in traits has been used as evidence for natural selection, and temperature is often proposed as the main driver of latitudinal clines in traits and allelic frequencies (Endler 1977). Clinal distributions therefore provide an opportunity to investigate the physiological mechanisms and genetic basis of ongoing thermal stress adaptation (Hoffmann 2010).

Latitudinal differentiation of *D. melanogaster* occurs for numerous life-history, morphological and stress resistance traits, and for many genetic markers that include the heat stress genes *hsp70*, *hsr-omega*, and small *hsps* (Hoffmann and Weeks 2007). While the exact nature of thermal selection that underpins latitudinal clines is not fully understood, the thermal stress experienced by *D. melanogaster* populations across the climatic regions is known to be different. Temperatures experienced by tropical populations are on average higher than temperate populations and tropical environments are more stable than the cooler and highly variable temperate environments in which hot and cold extremes are experienced more frequently (Ghalambor *et al.* 2006; Hoffmann 2010). Thermal plasticity, the internal physiological changes that improves survival and reproductive performance under stressful temperatures, is the major way that ectothermic species are adapted to periodic temperature stress. Whether stable tropical or fluctuating temperate environments have the greatest capacity for plasticity of thermal resistance traits is still unclear - the question is well researched in *Drosophila* (Trotta *et al.* 2006;

Overgaard *et al.* 2011; Cooper *et al.* 2012) and of ongoing general interest (Klopfer and MacArthur 1960; Brattstrom 1970; West-Eberhard 1989; Chown and Terblanche 2007). The plasticity of thermal-tolerance traits means that both culture temperature and assay conditions influence the strength of latitudinal patterns (Hoffmann *et al.* 2005; Terblanche *et al.* 2011). In fact latitudinal clines in adult *Drosophila* heat-knockdown tolerance are stronger after a brief hardening heat stress and the cline disappears altogether when dynamic assay conditions using natural rates of temperature increase are used (Sgrò *et al.* 2010). Certainly, rearing and testing conditions need to be taken into account when assessing clinal changes in thermal tolerance.

Despite the numerous associations of genetic markers with latitude and thermal tolerance in *D. melanogaster* (Hoffmann *et al.* 2003; Kolaczkowski *et al.* 2011) we have limited insight into the cellular and physiological mechanisms that underlie such variation. One study suggests that metabolic rate may be an important physiological trait involved with climatic adaptation, particularly under cooler growth conditions (Berrigan and Partridge 1997). However, recent application of genomic technologies to understanding the genetic basis of thermal tolerance variation indicate that many candidate genes fall into the 'GO'-groupings of *translation* and *regulation of transcription* (Leemans *et al.* 2000; Sørensen *et al.* 2005; Laayouni *et al.* 2007) suggesting that protein synthesis may be a relevant underlying process. During the cellular heat stress response the RNA expression of many hundreds of genes are up- or down-regulated following heat stress (Kültz 2005; Sørensen *et al.* 2005). However, in *Drosophila* within the first two hours following mild heat stress changes in levels of protein synthesis can be attributed largely to the production of new heat shock proteins (Hsps) and the curtailing of synthesis of normal cellular protein (the 25 °C proteins; Lindquist 1980; Storti *et al.* 1980). In fact early studies of heat tolerant strains of *D. melanogaster* demonstrate that changes in these processes are important to heat tolerance (Stephanou *et al.* 1983). Furthermore, a recent association between variation in heat hardening capacity and levels of total protein synthesis following a heat-stimulus support this idea (Johnson *et al.* 2009b). We know that Hsps are quickly up-regulated following a mild heat stress, that they have diverse modes of action (Feder and

Hofmann 1999), and that they are involved in protecting the cell from damage caused by heat (Parsell and Lindquist 1993). While numerous mechanisms that connect heat-stimulated protein translation with heat tolerance are possible, the simplest idea is that faster synthesis of Hsps quickly protect from cell damage and increase heat knockdown tolerance.

Both heat tolerance and protein synthesis levels in *D. melanogaster* have been associated with variation in the heat stress RNA gene *hsr-omega* (Rako *et al.* 2007; Johnson *et al.* 2011) that, like the *hsps*, is quickly up-regulated following heat stress (Pardue *et al.* 1990). This single copy gene contains two interesting polymorphic sites, at either end of the gene, and allelic variation at these two sites is largely independent in natural populations. Allelic frequencies at both sites cline with latitude and both are associated with thermal tolerance variation (Anderson *et al.* 2003; Collinge *et al.* 2008). Laboratory selection for high hardened heat tolerance each generation produced both heat tolerant populations and large replicated changes in expression levels of the two major *hsr-omega* transcripts (McKechnie *et al.* 1998). Furthermore, an increase occurred in the frequency of one allele that is more common in tropical populations (McColl *et al.* 1996; Anderson *et al.* 2003). The data strongly suggest that *hsr-omega* is a component of the hardened heat tolerance mechanism in this species. We now have some understanding of how this gene functions. *Hsr-omega* has the potential to influence general processing of diverse RNA transcripts and affect many cellular processes (Lakhotia 2011), particularly levels of total protein synthesis (Johnson *et al.* 2011). Low levels of the nuclear-located *omega-n* transcript associate with high basal rates of protein synthesis (Johnson *et al.* 2009a). *Omega-n* that is present in nearly all tissues is quickly up-regulated following heat shock and binds with crucial nuclear processing factors, an action thought to remove them from their normal role of intron processing. As a consequence intron-containing *mRNA* fails to mature and enter the cytoplasm for translation. This would reduce the synthesis of normal 25 °C proteins (that denature and aggregate under heat stress), and as a result more ribosomes would become available for faster production of protective chaperone proteins. Improved thermal performance might be expected because the energy for protein synthesis is conserved, faster production of Hsps would occur,

and reduced levels of any heat denatured 25 °C proteins would interfere less with normal cellular activities (Goldberg 2003).

The accumulated data and theory connecting *Drosophila* thermal tolerance, protein synthesis, and *hsr-omega*, is intriguing and invites further investigation. However the complexity of the cellular heat shock response, and the diverse nature of reported associations, generates numerous hypotheses about underlying cellular and physiological mechanisms. Here, to clarify relationships we take an exploratory approach at the population level and look for latitudinal clinal variation across climatic regions for all three traits in *D. melanogaster*. To increase the chances of detecting clinal patterns we use several testing/culture conditions. We compare clinal patterns for basal and heat-hardened knockdown tolerance in flies reared under two thermal regimes, 18 °C and 25 °C, approximating winter and summer growth conditions respectively. We know from previous research that heat resistance shows clinal variation (Hoffmann *et al.* 2002), but clinal populations have not previously been examined for hardened heat tolerance other than at 25 °C (Sgrò *et al.* 2010), although latitudinal clines in adult heat tolerance have been found under cooler and fluctuating rearing temperatures (Hoffmann *et al.* 2005). We also characterize clinal populations for protein synthesis levels both before and following a mild heat stimulus, and after rearing at both 18 °C and 25 °C. Finally we ask if expression levels of *omega-n*, the predominant transcript of *hsr-omega*, measured before and after a mild heat stimulus shows any latitudinal pattern of variation. Several interesting patterns emerge and we discuss possible ways that the patterns may relate to thermal tolerance and other fitness traits that vary across climatic gradients.

2.3 Materials and methods

2.3.1 Collection and maintenance of D. melanogaster

Two sets of populations were used in this study, one collected in 2008 and one in 2009. The 2008 collection was from 18 locations along a latitudinal gradient on the Australian east coast as reported by Sgrò *et al.* (2010). The 2008 collection was used for the heat tolerance and protein synthesis estimations. Each mass-bred laboratory population was generated from 30 isofemale lines from that location and maintained at population sizes of at least 500 flies per generation on potato-yeast-dextrose-agar medium in a 12:12 light: dark cycle, both at constant 25 °C and separately at constant 18 °C, for at least four generations prior to testing. Heat knockdown and protein synthesis experiments were performed on F_7 individuals.

For *omega-n* transcript level quantification, *D. melanogaster* populations were collected from eight points along a latitudinal gradient along the east coast of Australia (Northern Tasmania 41.24 °S, Melbourne 37.78 °S, Gosford 33.29 °S, Coffs Harbour 30.38 °S, Maryborough 25.54 °S, Rockhampton 23.45 °S, Bowen 19.98 °S, Innisfail 17.53 °S) in February 2009 (the 2009 collection). At least ten isofemale lines were established for each location and following three generations of laboratory culture two genetically independent populations were generated by combining five different isofemale lines for each location. Populations were maintained at population sizes of at least 500 flies each generation on potato-yeast-dextrose-agar medium. Experiments were performed after four generations of laboratory culture in a 12:12 light: dark cycle at constant 25 °C.

2.3.2 Heat tolerance

Prior to all assessments, population densities were controlled by counting and transferring 50 eggs per vial one generation prior to testing and by timed lays for the grand-parental and great-

grandparental generations. Adult basal and hardened heat knockdown tolerance was assessed on 5-7 day old females reared at a constant 25 °C (approximating summer development), or at a constant 18 °C (approximating winter development), since patterns change according to culture conditions (Hoffmann *et al.* 2005). Hardening involved exposing flies to 37 °C for 1 h, followed by 6 h recovery at 25 °C prior to testing. For each treatment (hardened and basal, both at 18 °C and 25 °C) 30 females were tested randomly over 10 runs over two days, with all runs containing equal numbers of each treatment. To assess knockdown time, individual flies were placed into water-tight 5 ml glass vials and submerged into a recirculating water bath held at 39 °C. Heat knockdown time was measured as the time taken for each fly to be immobilised from the heat. The heat knockdown assessment for the 2008 collection was carried out in parallel on the 25 °C- and 18 °C-reared populations. Note that the 25 °C data reported here (but not the 18 °C data) are those previously reported by Sgrò *et al.* (2010). The 25 °C data are included here so that I could explicitly examine the effect of rearing temperature on heat tolerance.

2.3.3 Protein synthesis

Rate of synthesis of total protein was measured in adult *Drosophila* ovaries by quantifying the amount of ^{35}S-labelled methionine incorporated into newly synthesised proteins in 1 h at 25 °C as per Johnson *et al.* (2009a); Johnson *et al.* (2011). Four treatments were tested, with total protein synthesis being measured in ovaries dissected from un-treated females reared at 18 °C, untreated reared at 25 °C, heat-stimulated females (1 h at 37 °C immediately prior to dissection) reared at 18 °C, and heat-stimulated females reared at 25 °C. For the heat stimulus females were placed in a 1.7 ml microcentrifuge tube with a pierced lid for ventilation and incubated in a water bath at 37 °C for 60 min. Immediately after heat stimulus flies were anaesthetised with CO_2 and ovaries dissected out for labelling. Samples (ovaries from 4 females) were incubated for 1 h in 6 µl Grace's Insect Medium (Invitrogen) and 6 µCi of ^{35}S-methionine (Amersham Biosciences). Following thorough washing to remove residual unincorporated label and subsequent extraction of proteins, counts per minute (CPM) of radioactive emissions from a supernatant aliquot of each

sample were recorded. Three replicates from each of 18 populations were assessed for each rearing temperature and each heat treatment. Populations were processed randomly and for each an un-treated (basal) rate measurement always immediately preceded a heat-stimulus measurement of the same population. Over six consecutive days, one block that contained all populations and both heat treatments was completed each day, with 25 °C-reared flies on the first three days and 18 °C-reared flies on the next three days (therefore limiting the comparison of protein synthesis rate between rearing temperatures). Data were corrected for day effects within rearing temperature by multiplying each radiolabel counts per minute (CPM) value by grand mean/run mean. To compare protein synthesis rates across populations that are known to vary latitudinally in body size (Lee *et al.* 2011a), incorporated label was expressed as CPM/gm of female after confirming, a) that female body weight differed significantly between populations (data not shown), and b) a positive association between total ovary protein content and female body weight at both rearing temperatures (data not shown). We used ovarian synthesis as a proxy for whole organism rates since ovaries represent a large proportion of body mass in mature females. Greater than 75% of our CPM measurement was due to labelled protein synthesis and not background noise (i.e. unincorporated label; see Johnson *et al.* 2011).

2.3.4 Omega-n *transcript quantification*

Levels of *omega-n* transcript were quantified in adults from eight populations (2009 collection), all reared at 25 °C, both before and after heat stimulus treatment (37 °C 1 h followed by 6 h recovery at 25 °C), using real time reverse transcriptase (RT)-PCR as per Collinge *et al.* (2008). For each population, three replicate RNA extractions of 20 adult female flies per treatment were obtained using TriSure reagent (Bioline, Alexandria, New South Wales, Australia). Genomic DNA contaminants were removed via incubation with Dnase (New England Biolabs). Reverse transcription was completed with 2 mg of total RNA primed with 200 ng random primers (Invitrogen, Mt. Waverley, Victoria, Australia) and using BioScript reverse transcriptase (Bioline) and the cDNA diluted by 1 in 10. Real-time PCR was performed in the LightCycler®

480 (Roche) system as per Lee *et al.* (2011a). PCR reactions were set up with 50% universal buffer (manufacturer's instructions, LightCycler® 480 High Resolution Melting Master, Roche), 10 µg cDNA (1% of reaction) and 400 nM (0.4% of reaction each) *omega-n*- and *cyclin K*-specific primers (*omega-n:* F- 5'-TCC GCA TTT ATT TTT CTC CAC-3', R-5'-GTG TAT AGA ATT TGG GAC CTC CA-3', *cyclin K*: F-5'- GAG CAT CCT TAC ACC TTT CTC CT-3', R-5'-TAA TCT CCG GCT CCC ACT G-3'; Collinge *et al*. 2008). Amplification conditions were 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s. Fluorescent data was acquired after the 72 °C step every cycle. Four technical replicates were performed per biological replicate. *Omega-n* transcript measures were internally normalized to *cyclin K* levels. To compare transcripts between samples, levels were established by obtaining cycle threshold (Ct) differences between *cyclin K* and *omega-n* values for each RNA extract and then converting to a fold difference value (Schmittgen and Livak 2008).

2.3.5 Data analysis

To test for differences between populations and any effects of rearing temperature and treatment on heat tolerance, protein synthesis and *omega-n* transcript levels, three- and two-way analyses of variance (ANOVAs) were performed. Treatment, population, rearing temperature and run were treated as fixed effects. Interactions between treatment, population and rearing temperature were also examined. Regression analyses were performed to test for latitudinal clinal variation in all traits examined. All analyses were performed using SPSS for Windows (IBM PASW Statistics 18.0.1). For the protein synthesis estimates some population samples were outliers (a standard score > +/- 2.5, as determined in SPSS) and these were routinely excluded, resulting in less than 18 populations in regression analyses.

2.4 Results

2.4.1 Heat knockdown time

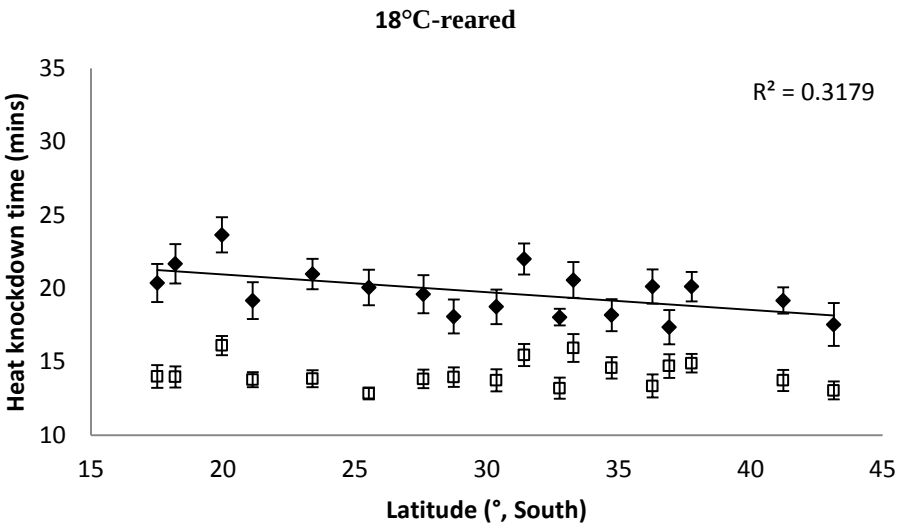
Rearing temperature and hardening treatment had significant effects on heat tolerance (Table 2.1). Rearing at 25 °C increased heat tolerance by 1.5 fold on average above flies reared at 18 °C. A significant effect of population on heat knockdown time was also detected, and all of the two- and three-way interactions were significant. Significant interaction between rearing temperature and hardening treatment reflects that following 18 °C rearing the average increase in knockdown time after hardening was 5.6 min, whereas this difference was increased to 8.1 min when reared at 25 °C. However, if you consider the relative changes in the effect of hardening, both rearing temperatures result in a 1.38 fold increase in heat knockdown time on average above basal time (Fig. 2.1). The significant population by rearing temperature interaction indicated that populations differed in their response to rearing temperature, illustrated by a difference of ~ 6 min between some populations in the extent of increase in heat knockdown time achieved when reared at 18 °C compared to 25 °C.

Regression analyses revealed a significant latitudinal cline in hardened heat knockdown time when flies were reared at both 18 °C and 25 °C - tropical populations were more heat tolerant after heat hardening than temperate populations (Fig. 2.1, Table 2.2). No indication of a cline was evident following culture at 18 °C. However, note the marginally non-significant cline in basal knockdown tolerance following 25 °C rearing (Fig. 2.1b, Table 2.2).

Table 2.1: Analyses of variance testing heat knockdown time, protein synthesis (reared at 18 °C and 25 °C) and *omega-n* transcript level for effects of population, rearing temperature and heat treatments. Knockdown time and protein synthesis measurements were using the 2008 collection of populations and *omega-n* transcript using the 2009 collection.

Trait	**Source**	**df**	**SS**	***F***	***p*-value**
Heat knockdown time (mins)	Rearing temperature	1	33560.17	1326.89	<0.0001
	Hardening treatment	1	22417.11	886.32	<0.0001
	Population	17	2881.66	6.70	<0.0001
	Run	9	2098.03	9.22	<0.0001
	Rearing temperature x Hardening treatment	1	691.41	27.34	<0.0001
	Rearing temperature x Population	17	1442.68	3.36	<0.0001
	Hardening treatment x Population	17	754.24	1.75	0.0285
	Rearing temperature x Hardening treatment x Population	17	832.86	1.94	0.0120
	Error	1904	48156.79		
Protein synthesis rate (18 °C)	Heat treatment	1	0.20	0.00	0.9829
	Population	17	13105.21	1.84	0.0421
	Heat treatment x Population	15	6060.87	0.96	0.5017
	Error	63	26394.29		
Protein synthesis rate (25 °C)	Heat treatment	1	1222.01	2.23	0.1418
	Population	13	17047.62	2.39	0.0134
	Heat treatment x Population	14	4220.65	0.64	0.7979
	Error	52	28557.02		
Omega-n transcript level	Population	15	1382.86	5.30	<0.0001
	Heat treatment	1	1646.19	94.62	<0.0001
	Population x Heat treatment	15	1283.80	4.92	<0.0001
	Error	60	1043.85		

a



b

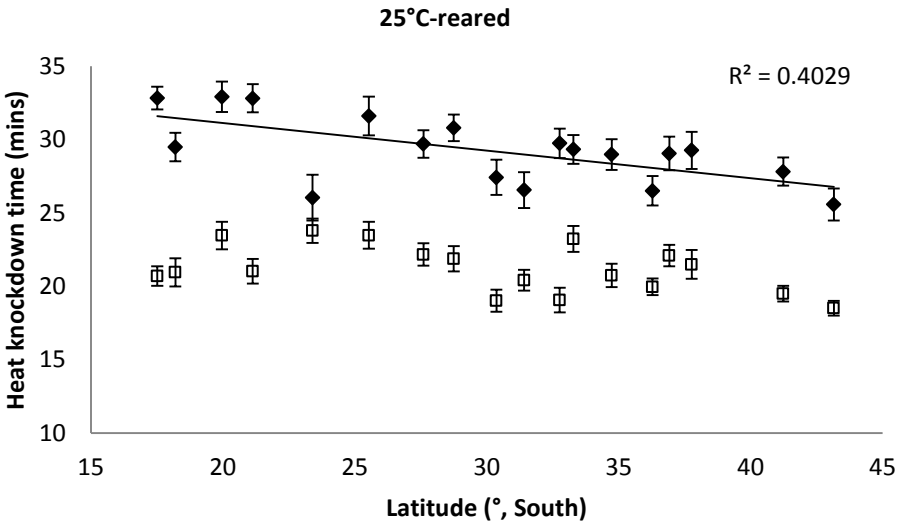


Figure 2.1 Latitudinal patterns of heat tolerance in *D. melanogaster* from the Australian east coast (2008 collection). Open squares represent basal knockdown time and filled diamonds represent hardened heat knockdown time. Error bars ± standard error. (a) Basal and hardened heat knockdown time when reared at 18 °C, (b) basal and hardened heat knockdown time when reared at 25 °C.

Table 2.2 Regression analyses for relationships between latitude and heat knockdown time, latitude and protein synthesis rates and latitude and *omega-n* transcript level in 18 °C- and 25 °C-reared *D. melanogaster* populations. Population means are used as data points in the analyses. Knockdown time and protein synthesis measurements were using the 2008 collection of populations and *omega-n* transcript using the 2009 collection.

Trait	**Rearing temperature**	**Treatment**	R^2	**b ± SE**	***t***	***p*-value**
Heat knockdown	18 °C	Basal	0.0077	-0.0106 ± 0.030	-0.35	0.729
	18 °C	Hardened	0.3179	-0.1207 ± 0.044	-2.73	0.015
	25 °C	Basal	0.2091	-0.0960 ± 0.047	-2.06	0.056
	25 °C	Hardened	0.4029	-0.1888 ± 0.057	-3.29	0.005
Protein synthesis	18 °C	Basal	0.3970	-1.3491 ± 0.444	-3.04	0.009
	18 °C	Heat-stimulated	0.0529	0.3489 ± 0.369	0.94	0.359
	25 °C	Basal	0.0139	-0.2538 ± 0.618	-0.41	0.688
	25 °C	Heat-stimulated	0.5500	-1.2470 ± 0.340	-3.67	0.004
ω-n transcript	25 °C	Basal	0.3112	0.2706 ± 0.108	2.52	0.025
	25 °C	Heat-stimulated	0.0183	-0.0858 ± 0.257	-0.33	0.749

2.4.2 Protein synthesis rates

Significant variation among populations was detected in the rate at which proteins were synthesised, when basal and heat-stimulated data were assessed as a whole, following rearing at both 18 °C and 25 °C (Table 2.1, Fig. 2.2). However heat stimulus treatment did not have a significant effect, and there was no significant interaction between population and heat treatment at either rearing temperature (Table 2.1).

Regression analyses revealed a significant negative latitudinal cline in basal rates of total protein synthesis when populations were reared at 18 °C - tropical populations showed faster rates of protein synthesis compared to their temperate counterparts (Fig. 2.2a, Table 2.2). However, following 18 °C rearing heat-stimulated protein synthesis did not show any clinal variation (Fig. 2.2b, Table 2.2). In contrast, when rearing occurred at 25 °C a cline was not detected for basal protein synthesis (Fig. 2.2c, Table 2.2), whereas a significant negative latitudinal cline was observed for heat-stimulated protein synthesis - tropical populations synthesised protein faster following heat stimulus than their temperate counterparts (Fig. 2.2d).

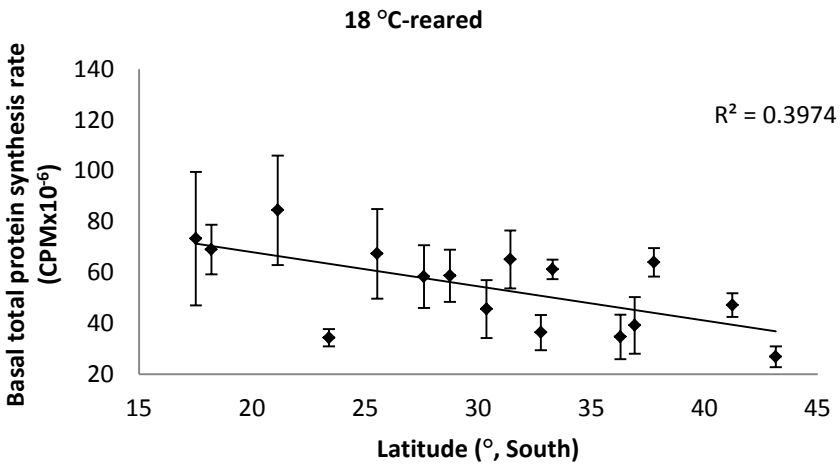
Among the populations from across the geographic range correlation between protein synthesis rate and heat tolerance revealed only two positive associations (Table 2.3). Following 18 °C-rearing an association between basal protein synthesis rate and hardened heat knockdown, and following 25 °C-rearing between heat-stimulated protein synthesis rate and hardened heat knockdown time.

2.4.3 Omega-n *transcript*

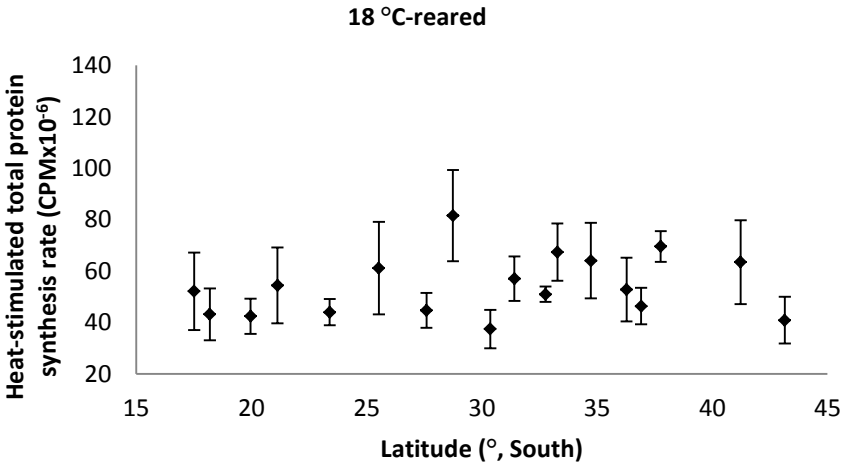
As expected, there was a significant effect of treatment - heat stimulated levels of *omega-n* transcript were higher than basal levels (Table 2.1, Fig. 2.3). There was also a significant effect of population, and a population by heat stimulus interaction (Table 2.1, Fig. 2.3). Significant

latitudinal patterns in *omega*-n transcript level were detected for both heat stimulated and non-heat stimulated (basal) flies. In non-heat stimulated flies a significant positive cline was observed - tropical populations had lower levels of transcript than temperate populations (Table 2.2). Interestingly, while no significant linear regression was detected for heat stimulated *omega-n* levels a non-linear parabolic clinal pattern was significant (R^2= 0.6593, intercept = -4.589±1.478, b = 0.077, *p*= 0.027; Fig. 2.3). Populations from either end of the cline had higher levels of *omega-n* than mid-latitude populations.

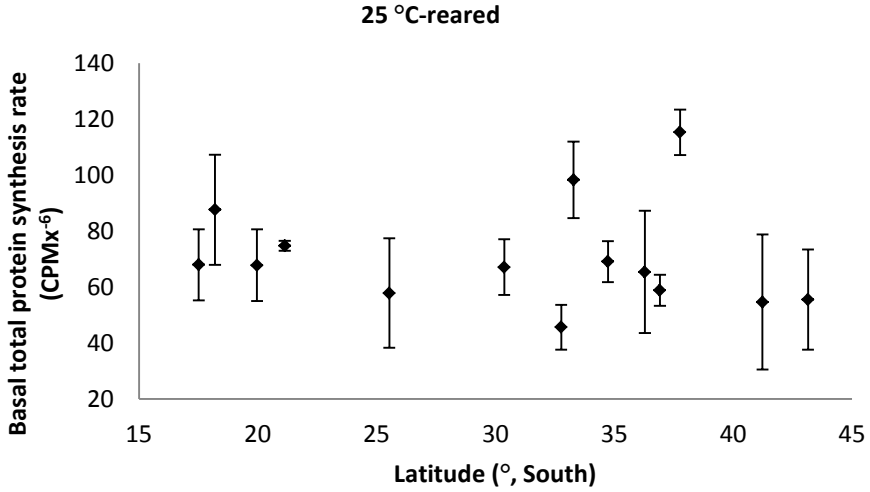
a


18 °C-reared
Basal total protein synthesis rate (CPMx10-6)
R² = 0.3974
140
120
100
80
60
40
20
15
20
25
30
35
40
45
Latitude (°, South)

b


18 °C-reared
Heat-stimulated total protein synthesis rate (CPMx10-6)
140
120
100
80
60
40
20
15
20
25
30
35
40
45
Latitude (°, South)

c


25 °C-reared
Basal total protein synthesis rate (CPMx-6)
140
120
100
80
60
40
20
15
20
25
30
35
40
45
Latitude (°, South)

d

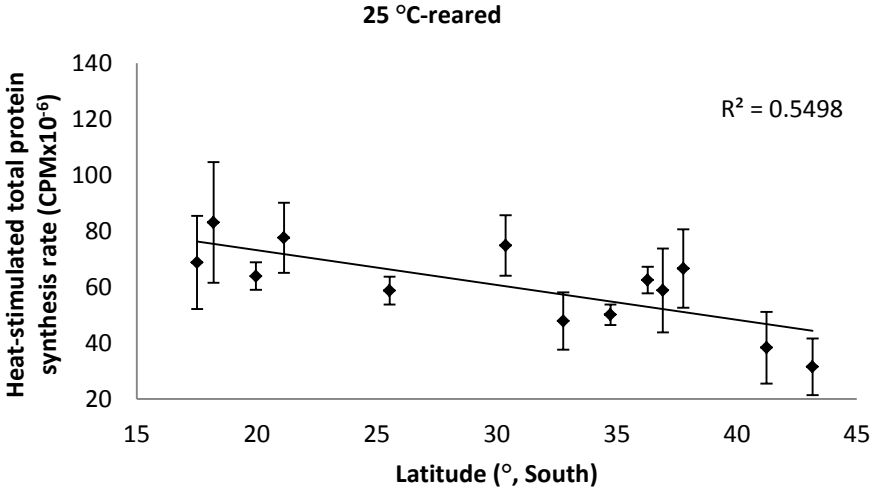

25 °C-reared
Heat-stimulated total protein synthesis rate (CPMx10-6)
R² = 0.5498
140
120
100
80
60
40
20
15
20
25
30
35
40
45
Latitude (°, South)

Figure 2.2: Latitudinal patterns of total protein synthesis rates in *D. melanogaster* females (2008 collection), (a) reared at 18 °C with no heat treatment, (b) reared at 18 °C with heat stimulus, (c) reared at 25 °C with no heat treatment, and (d) reared at 25 °C with heat stimulus. Error bars ± standard error.

Table 2.3 Associations between rates of protein synthesis and heat knockdown across the 2008 collection of populations of *D. melanogaster* reared at 18 °C and 25 °C.

Rearing temperature	**Protein synthesis measure**	**Heat tolerance measure**	***r***	**p-value**
18 °C	Basal	Basal	0.304	0.126
		Hardened	0.457	0.038*
25 °C	Basal	Basal	0.168	0.253
		Hardened	-0.152	0.274
18 °C	Heat-stimulated	Basal	0.383	0.088
		Hardened	0.109	0.356
25 °C	Heat-stimulated	Basal	0.357	0.116
		Hardened	0.476	0.050*

r: Pearson's correlation coefficients

Significance values are for one-tailed tests

*Significant p-values

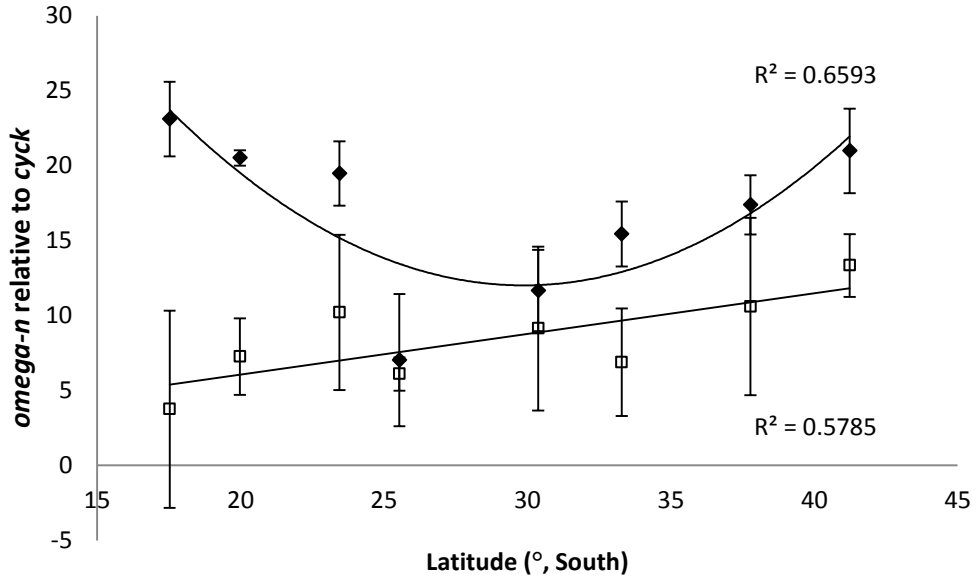


Figure 2.3 Latitudinal patterns of *omega-n* transcript level in 25 °C-reared populations (2009 collection) measured following heat stimulus (filled diamonds), or measured without heat stimulus (open squares). Error bars ± standard error.

2.5 Discussion

2.5.1 Heat tolerance

Our data confirm that rearing at cooler temperatures results in a decrease in heat tolerance in accordance with previous reports (Cavicchi *et al.* 1995; Hoffmann *et al.* 2005). The fact that about the same relative increase in knockdown time following heat hardening occurred after both cool- and warm-temperature rearing suggests that the hardening mechanism acts similarly but on the different levels of basal tolerance that occurred at each rearing temperature, somewhat like the relationship suggested from inter-specific comparisons (Kellett *et al.* 2005). While our data are consistent in direction with a negative latitudinal cline in basal heat knockdown tolerance in flies reared at 25 °C (Hoffmann *et al.* 2002), no cline in basal heat knockdown time was indicated following rearing at 18 °C. This is unexpected since heat knockdown clines persisted previously when populations were reared under simulated winter conditions (Hoffmann *et al.* 2005). Although possible, it seems unlikely that an evolutionary change has occurred in the seven years between field samples for these studies. Genetic differences underlying geographic patterns can depend on rearing temperature, as has been demonstrated for thoracic trident variation in *D. melanogaster* (Telonis-Scott *et al.* 2011). However, new field sampling and heat knockdown testing under cool rearing conditions will need to be re-assessed before reaching any conclusions about contemporary absence of a heat tolerance cline following cool-temperature rearing. Nonetheless in our current data strong clines were apparent in hardened heat knockdown time after rearing at both 18 °C and 25 °C.

2.5.2 Rates of protein synthesis

Our measure of total protein synthesis following mild heat stimulus (in ovarian tissue at 25 °C during the 1 h recovery following 1 h exposure to 37 °C) is a compound one. Previous work has shown that the cellular heat-shock response quickly activates during a 37 °C heat exposure

(Lindquist 1980), rapidly up-regulating synthesis of heat shock proteins (Hsps) and curtailing synthesis of normal cellular proteins (25 °C proteins). During recovery from the heat stimulus (when total protein synthesis was measured in this study), synthesis of Hsps (one of which is Hsp70) continues, diminishing slowly, and resynthesis of the normal 25 °C proteins begins to be restored (Storti *et al.* 1980; DiDomenico *et al.* 1982b; Stephanou *et al.* 1983) – the functional mRNAs of the 25 °C proteins having been preserved during the mild stress (Yost *et al.* 1990). Gel labelling intensity suggests, conservatively, that at least two-thirds of the synthesis during the recovery-hour is of Hsps, mostly Hsp70. Such complexity is not relevant when basal (non-heat stimulated) levels of protein synthesis are measured. Also, while this measure of ovarian total protein synthesis seems a reasonable proxy for rates of synthesis in the whole fly (in mature females ovarian tissue constitutes a high proportion of total body mass) we need to be aware that it may not reflect patterns of protein synthesis in other body tissues. Nonetheless it seems reasonable that estimates from ovarian tissues provide insight into patterns of change in protein synthesis over a range of culture conditions and over a range of climatic regions.

As discussed, our experimental regime for measuring protein synthesis precluded us from determining if rearing temperature made a difference to levels of total protein synthesis (that was always measured at the same temperature). We used four 'treatments' to test for a latitudinal cline in rates of total protein synthesis, two of which revealed the presence of a cline. In both cases higher rates of total protein synthesis occurred in tropical populations. In the first, when populations developed under cooler conditions (18 °C), basal rates of protein synthesis were lower in populations from temperate regions. We might have expected a cline in the opposite direction given the normal physiological compensatory effects associated with adaptation to colder regions (Clarke 2006; Frazier *et al.* 2006). Perhaps the cline might reflect an adaptation of temperate populations to a change in reproductive strategy necessary to persist through a cold winter, when maturation of eggs as winter approaches is wasteful (Saunders and Gilbert 1990; Mitrovski and Hoffmann 2001). Basal protein synthesis was estimated in ovarian tissue and the lower rates under cooler conditions might reflect the winter slowdown in ovarian development,

including for example less yolk protein production (Bownes 1980). Such a strategy would not be relevant for tropical populations.

The second treatment to reveal a cline was when protein synthesis was measured following development under warm conditions (25 °C) and immediately after females experienced a mild heat stimulus. Given the complexity of protein synthesis changes following a heat stimulus future research should re-examine this cline using fine-grained physiological assays that identify synthesis rates of specific proteins, or protein classes. Future study should also examine protein synthesis in non-ovarian tissue. Nonetheless, we can speculate that if the higher rate of total protein synthesis following heat stimulus in tropical populations is due to faster Hsp production we might expect increased heat tolerance in tropical populations, as has been documented (Hoffmann *et al.* 2005) but not significantly so in this study. This expectation is based on the idea that faster Hsp synthesis leads to faster production of protective proteins that contributes to higher knockdown tolerance. We did observe higher heat-hardened tolerance in tropical populations, and weak indications of a direct positive association between protein synthesis and knockdown tolerance was obtained for the 18 populations sampled across the geographical transect (Table 2.3), so this idea should not be discarded.

In a recent study a robust negative association was detected between the levels of heat-stimulated total protein synthesis and absolute heat hardening capacity – families with higher levels of heat-stimulated protein synthesis had lower hardening capacity (Johnson *et al.* 2009b).Thus we might have expected tropical populations to have reduced hardening capacity and this has certainly not been observed – if anything the opposite tendency is noted (Fig. 2.1). A reason for the apparent discrepancy could be the geographical differences in the strains employed in the two studies and the issue is addressed in a separate investigation (Cockerell *et al.* 2013).

The presence of latitudinal patterns in rates of protein synthesis suggests that these are important physiological traits, and that they may underpin latitudinal differentiation of fitness traits other

than adult heat knockdown tolerance. This is particularly the case if our ovarian protein synthesis measurements reflect variation across all tissues of both sexes. Protein synthesis can influence developmental and fitness characteristics (Marygold *et al.* 2007), particularly under stress conditions (Teleman *et al.* 2005). High protein synthesis rate has been linked to poor longevity in the nematode, *C. elegans* (Syntichaki *et al.* 2007) and in several other species (reviewed in Tavernarakis 2008). In one intra-population study protein synthesis rate was not linked to development time, fecundity or body size in *Drosophila* (Johnson *et al.* 2009a), however this study used only a single population and had low statistical power. Given that protein synthesis rates were measured in ovaries it would be interesting to see how the clinal patterns relate to reproductive fitness traits that show climatic differentiation, such as egg size, ovariole number (Azevedo *et al.* 1996), egg production (Mitrovski and Hoffmann 2001), and productivity (Trotta *et al.* 2006).

2.5.3 Omega-n

The higher levels of *omega-n* following a mild heat stimulus were expected given the established heat-inducibility of this gene (Pardue *et al.* 1990). Our first novel finding for *hsr-omega* is that the Australian tropical populations had lower basal levels of the nuclear transcript than temperate populations. *Omega-n* transcript level influences rates of protein synthesis, with low basal levels of *omega-n* resulting in higher rates of protein synthesis (Johnson *et al.* 2011). We might therefore expect tropical populations to show higher basal levels of protein synthesis and in general this was the case, but only under cool culture conditions or following heat-stimulus that up-regulates the transcript. Since the *omega-n* transcript data were collected only for the 2009 populations we are unable to test here for a direct relationship with protein synthesis or tolerance. However since basal *omega-n* levels were measured following culture at 25 °C, conditions that did not reveal a cline in protein synthesis, we found no indirect relationship between these exact variables. Our second novel *hsr-omega* finding is that heat-stimulated levels of *omega-n* followed a non-linear parabolic geographical pattern. While parabolic latitudinal

clines in allelic frequencies or gene expression patterns are not necessarily linked directly to specific trait variations, in this and other *Drosophila* species several traits and allele frequencies are known to vary latitudinally in a non-linear fashion (Hallas *et al.* 2002; Magiafoglou *et al.* 2002; Sgro and Blows 2003; Sarup *et al.* 2006; Umina *et al.* 2006; van Heerwaarden *et al.* 2012). Also, at least one trait is predicted to vary non-linearly with temperature (David *et al.* 2003). These non-random patterns are of interest since they provide clues about thresholds for underlying physiological processes or clues about adaptive processes that operate differentially across environmental gradients.

Since the level and length of *omega-n* varies clinally (Collinge *et al.* 2008)(Collinge *et al*. 2008), since the levels of *omega-n* affect protein synthesis, and since heat-stimulated protein synthesis did show clinal variation under particular conditions, a link between *omega-n* transcript level and protein synthesis in the clinal context needs to be further investigated. Such a study would need to be cognisant of at least two other factors. First, the polymorphic site at the distal end of the *hsr-omega* gene has many different alleles each differing in the number of copies of ~ 280 bp DNA-sequence repeat. Some copies of this gene in natural populations are as short as 8 kb in length, others as long as 21 kb as a consequence of variation in copy number of this repeat sequence (Hogan *et al*. 1995). Tropical populations have on average more of the RNA copies of these repeats in the *omega-n* transcript than temperate populations (Collinge *et al*. 2008), and these repeats are likely to be the functional units of the transcript (see Johnson *et al.* 2009a, and references therein). More repeats would be expected to help down regulate basal rates of protein synthesis, and produce lower levels of protein synthesis in the tropics - a prediction not generally upheld by our data. Also note that in the tropics the higher *omega-n* transcript levels measured after heat stimulus, compared to mid-latitude populations in the parabolic pattern, might also be expected to decrease heat-stimulated protein synthesis in these populations. The second confounding factor that needs to be considered is that this Australian latitudinal transect of populations covers a parallel cline for the common cosmopolitan inversion *In(3R)Payne* that is at high frequency in the tropics and carries many hundreds of genes, including *hsr-omega,* that are

in strong linkage disequilibrium (Anderson *et al.* 2005). While the *hsr-omega* repeat variation is largely independent of *In(3R)Payne* (Collinge *et al.* 2008), the other variable site within this gene, an 8 bp indel polymorphism, is in strong linkage disequilibrium with the inversion. The allele without the insert (*hsr-omega*^{S}) occurs in about 60% of *In(3R)Payne* chromosomes (Anderson *et al.* 2005), is at high frequency in the tropics, and has been associated with higher levels of the *omega-n* transcript (Johnson *et al.* 2009a), an observation that is puzzling given our clinal *omega-n* transcript data indicating lower levels in the tropics. Clearly the picture is complex and depends heavily on culture and testing conditions. A first step to clarify these issues would be to examine relationships between *omega-n* levels and rates of synthesis of Hsps, or shutdown of 25 °C proteins following mild heat stress, measured separately, both in a single set of lines, and/or in a single collection of populations from the latitudinal transect. Controlling, experimentally or statistically, for the presence of *In(3R)Payne* would be an important part of the design.

Through its potential to affect rates of protein synthesis, as well as other influences on cellular activities (Lakhotia 2011), *omega-n* could influence many traits that vary latitudinally, such as cold tolerance (Collinge *et al.* 2008), development time (James and Partridge 1995), or male fertility (Rako *et al.* 2009), assuming variation in our *omega-n* measurements in extracts from whole females reflects clinal patterns in other tissues of both sexes. In fact *hsr-omega*, given its genotype and expression associations with both latitude and thermal tolerance, rather than having a major influence on heat knockdown time, may influence post-heat-stress reproductive traits such as male fertility and female egg production, particularly given the high levels of *omega-n* reported in *Drosophila* testes and ovaries (Mutsuddi and Lakhotia 1995).

2.5.4 Conclusion

This study demonstrates the importance of rearing temperature and heat-hardening treatment on the pattern and strength of clinal variation in adult *D. melanogaster* heat tolerance. In this species

we provide new evidence that total protein synthesis rates vary with latitude, and for the first time we describe two interesting patterns of clinal variation in levels of the nuclear transcript of the heat-stress RNA gene *hsr-omega*. In this exploratory project our data have led to more questions than answers. The direction of the clines in the three traits are consistent with a simplified underlying causal explanation, linking high heat tolerance in the tropics with faster general synthesis of heat shock proteins and lower levels of *omega-n* that could be expected to increase protein synthesis. However we have not provided additional evidence to support this hypothesis since only a low level of direct positive association was detected between ovarian-protein synthesis and our measures of heat tolerance. Any renewed approach should re-examine these clines and use finer-grained physiological assays to characterise variation in specific proteins or protein classes. Nonetheless the non-random latitudinal patterns of rates of protein synthesis and *hsr-omega* transcript levels are likely to lead to changes in other traits that are locally adaptive. A future challenge is to identify such geographically variable traits.

2.6 Acknowledgements

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Monash University

Declaration for Thesis Chapter 3

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	Extent of contribution (%)
Design, research, analysis, manuscript preparation	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	Extent of contribution (%) for student co-authors only
Carla Sgrò	Analysis	5%
Stephen McKechnie	Design, manuscript preparation	25%

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**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

CHAPTER 3

Capacity for protein synthesis following heat stimulus of *Drosophila* associates with heat tolerance but does not underlie the latitudinal tolerance cline

3.1 Abstract

Across populations of *D. melanogaster* along the Australian eastern coastline latitudinal clines occur in both heat-knockdown tolerance and hardened heat-knockdown tolerance – low latitude tropical populations being more tolerant. A latitudinal cline also occurs for rates of total protein synthesis following a mild heat stress, with tropical populations having higher rates. Since the control of protein synthesis following heat stress is an important component of the cellular heat-shock response, we hypothesised that the higher rates of synthesis that follow a heat stimulus lead to higher knockdown tolerance and underpins the cline. However, levels of heat-stimulated total protein synthesis have been negatively related to heat-hardening capacity, a somewhat conflicting result. Here we examine the relationship between these physiological and adaptive traits in a set of 40 family lines derived from a hybrid laboratory population established by crossing populations from either end of the latitudinal transect. Among these lines high levels of heat-stimulated total protein synthesis were associated with both low basal and low heat-hardened adult knockdown time, confirming the importance of a negative relationship between protein synthesis and thermal tolerance. This result, when considered along with the directions of the latitudinal clines in protein synthesis and tolerance, suggests that variation in rates of heat-stimulated total protein synthesis is not a factor contributing to the latitudinal cline in heat tolerance. Given the robustness of this negative relationship we discuss possible explanations and future experiments to elucidate how the cellular heat stress response might facilitate increased knockdown tolerance.

3.2 Introduction

Heat stress affects many organisms across the globe, with changing climates posing a real threat to species' distributions and abundance. Now that we are overtly aware of global warming increased effort has been put towards understanding adaptation to warmer environments (Dillon *et al.* 2010; Hoffmann and Sgrò 2011). Intra-specific thermal tolerance variation among populations and strains has been a focus for investigation of the selective and underlying physiological and genetic basis for such adaptive change, especially in ectothermic species such as fish and insects (Deutsch *et al.* 2008; Healy and Schulte 2012). In particular, adaptive geographic variation in heat tolerance in numerous species shows how populations evolve to suit the thermal environment (Bahrndorff *et al.* 2006; Sarup *et al.* 2006; Kuo and Sanford 2009).

The thermal tolerance clines across latitudinal gradients in *Drosophila melanogaster* have been well characterised (Guerra *et al.* 1997; Hoffmann *et al.* 2002; Fallis *et al.* 2011). Several other traits also cline incrementally with latitude along the Australian eastern coast in this species (Hoffmann and Weeks 2007), and they are underpinned by genetic differentiation established by differential selective processes in divergent climatic regions (Kolaczkowski *et al.* 2011). The three heat tolerance traits that cline are adult heat knockdown time, heat-hardened knockdown time, and heat-hardening capacity (Hoffmann *et al.* 2005; Sgrò *et al.* 2010; Cockerell *et al.* 2014). Testing conditions are important however as not all measures of heat tolerance show clinal variation (Sgrò *et al.* 2010; Terblanche *et al.* 2011).

Recent progress towards understanding the physiological basis of heat tolerance variation in *D. melanogaster* suggests that heritable variation in levels of protein synthesis may be important. First, recent application of genomic technologies to understanding the genetic basis of the response to heat stress indicate that many candidate genes fall into the 'GO'-groupings of *translation* and *regulation of transcription* (Leemans *et al.* 2000; Sørensen *et al.* 2005; Laayouni *et al.* 2007), suggesting that protein synthesis may be a relevant underlying process. A change in

the control of protein synthesis following heat stress has been recognised for a number of years – it is an important component of the cellular heat-shock response (Storti *et al.* 1980; Ballinger and Pardue 1983) in which new heat shock proteins are synthesised and the synthesis of vulnerable housekeeping proteins is curtailed (Lindquist 1980; Sørensen *et al.* 2005). The association between *Drosophila* heat tolerance and enhanced synthesis of heat-shock proteins was first reported in the 1980s (Stephanou *et al.* 1983). The heat-shock proteins are a group of highly conserved proteins that act as molecular chaperones with numerous diverse functions that protect and stabilise other proteins, assisting with protein folding, re-naturation, transport and degradation (Parsell and Lindquist 1993). Numerous studies suggest that higher levels of the heat-inducible heat-shock proteins associate with higher thermal tolerance (Parsell and Lindquist 1993), however not all data are in agreement (Feder and Hofmann 1999; Jensen *et al.* 2010).

The simplest hypothesis that connects protein synthesis with heat tolerance is that faster synthesis of proteins that are up-regulated in the cellular heat-shock response would increase heat knockdown tolerance. The possibility that variation in protein synthesis may contribute to the Australian latitudinal clines in thermal tolerance is highlighted by the recent detection of clinal variation in levels of heat-stimulated protein synthesis along the same latitudinal transect (Cockerell *et al.* 2014). This parallel clinal variation represents an intriguing possibility for a causal connection between a physiological mechanism and an adaptive trait since higher rates of heat-stimulated protein synthesis were detected in low-latitude tropical populations that are associated with higher heat tolerance. However in this latter study only a low level of direct association was detected between rates of protein synthesis and adult heat knockdown tolerance.

In contrast, in studies of multiple family lines isolated from a single population, a negative association was detected between the rate of heat-stimulated protein synthesis and the extent of increase in knockdown time following a heat-hardening stress (Johnson *et al.* 2009b). The measure of protein synthesis used in both of these studies detects total new protein synthesised in the 60 min recovery period following a 60 min heat stimulus (37 °C). The cellular heat-shock

response quickly activates during such a mild heat pulse (Lindquist 1980), rapidly up-regulating synthesis of Heat shock proteins (Hsps) and curtailing synthesis of normal cellular proteins (25 °C proteins). During the second recovery hour, when total protein synthesis was measured, synthesis of Hsps (one of which is Hsp70) continues, diminishing slowly, and resynthesis of the normal 25 °C proteins begins to be restored (Storti *et al.* 1980; DiDomenico *et al.* 1982a; Stephanou *et al.* 1983). This measure of protein synthesise is therefore a compound one, since it confounds both of these protein synthesis processes.

The above two studies therefore provide somewhat conflicting information - the clinal data being consistent with the hypothesis that faster protein synthesis is positively related to higher heat tolerance, that may help explain the latitudinal clines in tolerance, and the family line data that suggests that a faster rate of heat-stimulated protein synthesis is negatively related to heat hardening capacity. Here we test the hypothesis of a positive protein synthesis – tolerance relationship and re-examine whether or not heat-stimulated protein synthesis is negatively related to *Drosophila* heat knockdown tolerance. The approach is similar to one previously used to maximise trait variation and to test for the robustness of correlations between stress resistance and other traits (Hoffmann *et al.* 2001). We perform a family association study that incorporates the broadest spectrum of genetic variants from across climatic regions. We derive a hybrid population by crossing two populations, one from the tropical end and one from the temperate end of the latitudinal cline. After mass rearing in the laboratory for twelve generations, to facilitate genome mixing and to minimise latitude specific linkage disequilibrium, we establish a set of 40 family lines and characterised them for variation in both adult heat knockdown tolerance and rates of protein synthesis. The associations we detect provide clear answers to our questions and strongly indicate that latitudinal variation in rates of protein synthesis do not contribute to clinal variation in thermal tolerance.

3.3 Materials and Methods

3.3.1 Collection and maintenance of lines

Protein synthesis rate and heat knockdown time were measured on a set of 40 single-family lines of *D. melanogaster* established by crossing northern and southern latitude populations. This set of hybrid lines contained gene combinations from the entire latitudinal cline (latitudinal populations used in Sgrò *et al.* 2010 and Cockerell *et al.* 2014) that were generated by disrupting gene combinations from the cline ends. First, one southern and one northern population were set up with equal numbers of adult flies from each of three southern populations (latitudes 43.15 °S, 37.77 °S and 36.92 °S) and three northern populations (19.97 °S, 18.20 °S, 17.52 °S), respectively. More than a hundred northern males and an equivalent number of southern virgin females, and the reciprocal cross, were mated for two days and then combined to form one large mass-bred population. After twelve generations as a single mass-bred population (maintained in four culture bottles each containing 80 ml medium - flies being mixed each generation and approximately 300 flies contributing to egg production for four hours per bottle, per generation) 240 lines were initiated using virgin females in single-pair crosses. These 240 lines were reduced to forty lines by performing heat tolerance tests on 10 males and 10 females from each line, using the protocol for heat tolerance testing described below, and selecting the 10 most and 10 least heat tolerant lines, from each of the heat hardened and basal tests, as a strategy to maximise the chances of finding an important physiological or genetic association. Lines were maintained in large numbers (as above) in single bottle culture on potato-yeast-dextrose-agar medium and experiments were performed on F_7 individuals. Density was controlled one generation prior to testing for both heat knockdown tolerance and rates of protein synthesis by having flies lay on medium on small spoons, and counting and transferring 50 eggs to single-vial cultures.

3.3.2 Rate of protein synthesis

Protein synthesis rate was measured in mature female ovaries by quantifying the amount of ^{35}S-labelled methionine incorporated into newly synthesised proteins in 1h at 25 °C as described in detail by Johnson *et al.* (2009b). This method was basically that reported by (Stephanou *et al.* 1983) except that live females, rather than excised ovaries, were heat stimulated. Briefly, for the heat stimulus, females were placed in a 1.7 ml microcentrifuge tube with a pierced lid for ventilation and incubated in a water bath at 37 °C for 60 min. Immediately flies were anaesthetised with CO_2 and ovaries dissected out for labelling. Samples (ovaries from 4 females) were incubated for 1 hr in 6 µl Grace's Insect Medium (Invitrogen) and 6 µCi of ^{35}S-methionine (Amersham Biosciences). Following thorough washing to remove residual unincorporated label and subsequent extraction of proteins, counts per minute (CPM) of radioactive emissions from a supernatant aliquot of each sample were recorded. Three replicate assays using untreated females and three replicate assays using heat-stimulated females were performed for each of the forty lines (a total of 240 samples were measured). Females for each assay were from different rearing vials. One untreated and one heat-stimulated biological replicate from each line was completed on each day. Within each block/day ten assays occurred per session (five lines per session, each non-treated assay being followed by a heat-stimulated assay of the same line). Lines and were selected at random in any session within a day until all three heat-stimulated and three untreated assays were completed for each line.

3.3.3 Heat tolerance

Adult basal and hardened heat knockdown tolerance was assessed in parallel for 5-7 day old females reared under constant 25 °C conditions (as in Cockerell *et al.* 2014). Hardening involved exposing flies to 37 °C for 1 h, with 6 h recovery at 25 °C prior to testing. To assess knockdown time, individual flies were placed into 5ml glass vials that were sealed water-tight with a plastic cap (many being held in a rack) and submerged into a recirculating water bath held at 39 °C.

Heat knockdown time was measured as the time taken for each fly to be rendered immobile from the heat. For each treatment (hardened and basal) 12 females per line were tested randomly over 10 runs over two days, with all runs containing equal numbers of each treatment.

3.3.4 Data analysis

Protein synthesis data (CPM values) were corrected for session effects by multiplying each by grand mean/session mean. We also calculated for each line the change in rate of protein synthesis following heat stress by subtracting the heat-stimulated rate from the basal rate. Heat knockdown time data were corrected for run by multiplying each knockdown time by grand mean/run mean. Hardening capacity was also determined for these data, calculated as hardened heat knockdown time minus basal heat knockdown time. To test for protein synthesis and knockdown time differences among lines, and interactions between heat treatments and line, two-way analyses of variance were performed. Treatment and line were treated as fixed effects. Linear regression analyses, Pearson correlation coefficients and partial correlations were used to look for relationship between protein synthesis and heat tolerance. All analyses were performed using SPSS for Windows (IBM PASW Statistics 18.0.1).

3.4 Results

Significant variation in protein synthesis rate was detected among lines (Fig. 3.1, Table 3.1). The heat stimulus reduced protein synthesis rate, which was on average 27% lower in flies that were heat-stimulated compared to those that were not. While not significant, a positive correlation occurred between basal and heat-stimulated protein synthesis ($r = 0.26$, $p = 0.058$). The presence of a significant interaction indicated that the extent of reduction in protein synthesis following heat stimulus was quite different for different lines (Fig. 3.1).

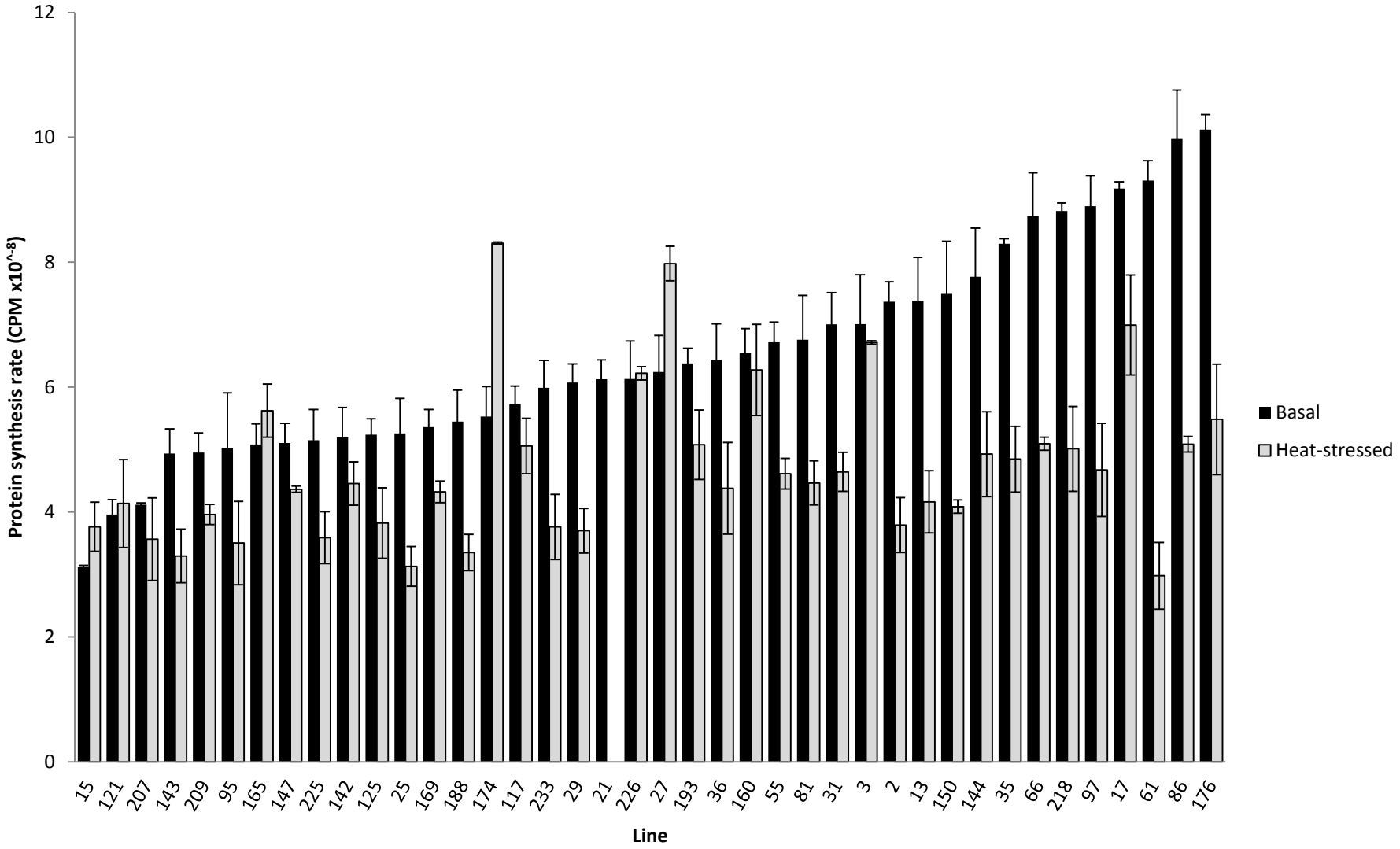

Protein synthesis rate (CPM x10^-8)
12
10
8
6
4
2
0
Basal
Heat-stressed
15
121
207
143
209
95
165
147
225
142
125
25
169
188
174
117
233
29
21
226
27
193
36
160
55
81
31
3
2
13
150
144
35
66
218
97
17
61
86
176
Line

Figure 3.1 Rates of basal and heat-stimulated total protein synthesis for the 40 lines from the hybrid population, ranked in order of ascending basal rates. Error bars ± standard error (n = 3 for each bar).

Table 3.1 Two-way analyses of variance testing for effects of line and heat stress on rates of ovarian protein synthesis and for effects of line and heat hardening on heat knockdown time across forty lines from the hybrid population.

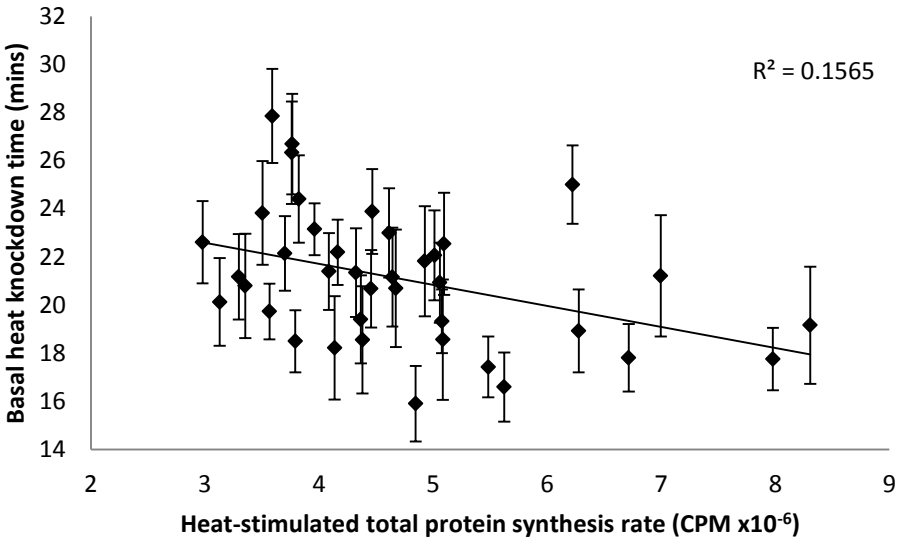
Trait	**Term**	**df**	**SS**	***F***	***p***
Protein synthesis	Line	39	321.87	11.803	<0.0001
	Heat-stress treatment	1	174.22	249.163	<0.0001
	Line x heat-stress treatment	39	201.20	7.378	<0.0001
	Error	158	110.48		
Heat knockdown	Line	40	7602.56	4.17	<0.0001
	Hardening treatment	1	7225.14	158.43	<0.0001
	Line x hardening treatment	37	1825.98	1.08	0.3417
	Error	819	37351		

Significant variation for both basal and hardened heat knockdown time was detected among lines, as indicated by two-way Anova (Table 3.1). No interaction was detected between line and hardening treatment suggesting that the heat hardening treatment had a similar effect on all lines, increasing knockdown time from an average of 20.9 mins to 26.5 mins (Fig. 3.2).

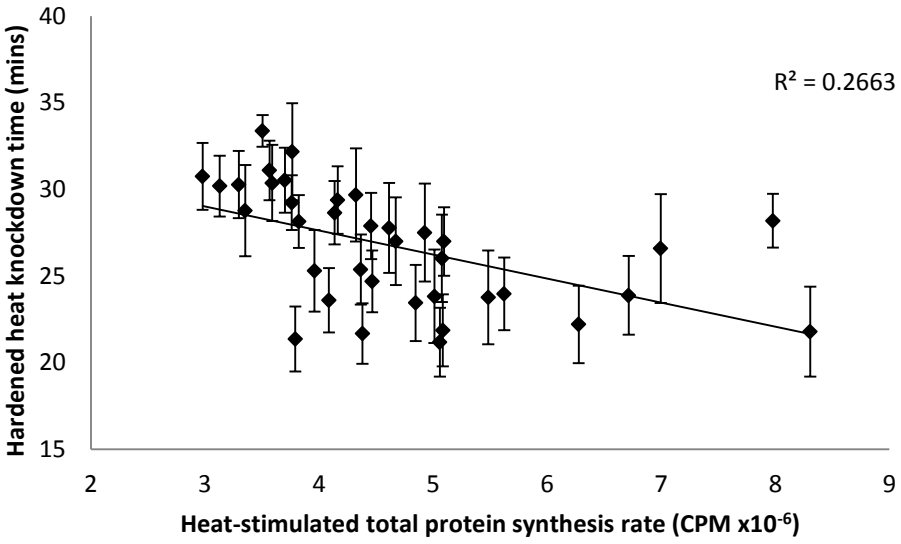
Heat-stimulated protein synthesis rates showed significant negative associations with both basal heat knockdown time and heat-hardened knockdown time (Table 3.2, Fig. 3.2), and basal rates of protein synthesis were negatively associated with hardened knockdown time. The partial correlation of basal rate of protein synthesis with hardened knockdown time, controlling for rate of heat-stimulated protein synthesis, reduced the association to a non-significant value (r_p = -0.297, p = 0.078), whereas the relationship of heat-stimulated protein synthesis to hardened knockdown time, controlling for basal rate of protein synthesis, remained significant (r_p = -0.469, p = 0.004). This suggests that the more robust association was between heat-stimulated protein synthesis and hardened knockdown time. No associations were detected between basal or heat-stimulated protein synthesis and heat hardening capacity (Table 3.2).

Given the marked variation among lines in the extent of difference between heat-stimulated and basal rates of protein synthesis we looked for associations between the rate differences and the heat tolerance measures, but none were indicated (data not shown).

a


Basal heat knockdown time (mins)
32
30
28
26
24
22
20
18
16
14
2
3
4
5
6
7
8
9
R² = 0.1565
Heat-stimulated total protein synthesis rate (CPM x10-6)

b


Hardened heat knockdown time (mins)
40
35
30
25
20
15
2
3
4
5
6
7
8
9
R² = 0.2663
Heat-stimulated total protein synthesis rate (CPM x10-6)

c

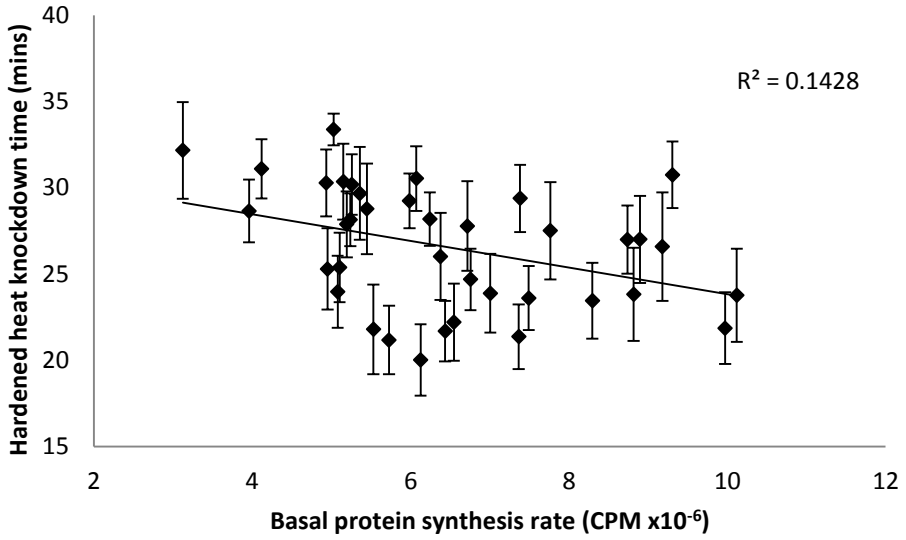


Figure 3.2 Relationships between protein synthesis rate and heat tolerance in lines from the hybrid population. (a) Basal knockdown time associated with heat-stimulated total protein synthesis rate ($p = 0.013$, slope = -0.873±0.333, $R^2 = 0.157$). (b) Hardened heat knockdown time associated with heat-stimulated total protein synthesis rate ($p = 0.001$, slope = -1.388±0.389, $R^2 = 0.266$). (c) Hardened heat knockdown time associates with basal protein synthesis rate ($p = 0.019$, slope = -0.771±0.315, $R^2 = 0.143$). Error bars ± standard error (based on 12 females per line).

3.5 Discussion

For technical expediency we have measured basal and heat-stimulated total protein synthesis in ovarian tissue as this seemed a reasonable proxy for rates of synthesis in the whole fly - in mature females the ovarian tissue constitutes a high proportion of total body mass. We therefore need to keep in mind that, while the cellular heat stress response occurs in most if not all tissues (Kültz 2005), this measure may not reflect exact patterns of protein synthesis in other body tissues.

In an earlier study, that used a set of lines derived from a single field population, a negative association was detected between heat-stimulated protein synthesis and hardening capacity (Johnson *et al.* 2009b). In the current set of lines this specific association was not detected. These hybrid lines did not show significant differences in heat hardening capacity which may be one reason why we did not detect a hardening capacity / protein synthesis association. However, among these hybrid lines negative relationships were detected between rates of protein synthesis, particularly after a heat stimulus, and adult heat knockdown time. Although basal levels of protein synthesis were negatively associated with hardened knockdown time it was heat-stimulated protein synthesis that was the more robustly negatively associated, with both basal and hardened knockdown time, since partial correlation analyses suggested that the association between basal rate of protein synthesis with knockdown time was a consequence of the relationship between basal and heat-stimulated rates of protein synthesis.

Consistent between the current study and that of Johnson *et al.* (2009b) is that the associations in both studies are negative in direction and both involve heat-stimulated protein synthesis. This negative relationship however does not help explain the latitudinal clines in knockdown time since *both* protein synthesis rate and knockdown time increase at tropical latitudes, and a positive relationship would be required. Therefore it seems likely that other physiological processes have an over-riding effect on any influence that protein-synthesis might have on the latitudinal cline in

adult heat knockdown time. While clinal latitudinal variation in protein synthesis in tissues of *D. melanogaster* does not obviously relate to clinal variation in adult heat knockdown tolerance, clines in rates of both heat-stimulated protein synthesis and basal protein synthesis (when cultured at 18 °C - Cockerell *et al.* 2014) might differentially affect cellular processes at different latitudes. This variation may influence other quantitative traits involved with climatic adaptation. Further investigation into the role of protein synthesis in climatic adaptation is therefore warranted, particularly how rates of heat-stimulated protein synthesis measured in ovaries relate to reproductive fitness traits measured following periods of thermal stress.

The extent to which total protein synthesis was reduced following the heat stimulus was notably different among the lines - a few lines even appeared to increase protein synthesis following the heat treatment. This suggested the idea that the more extreme changes are needed in some lines to maintain tolerance, i.e. that these are the less tolerant lines. However the absence of any association between the extent of heat-stimulated change in protein synthesis and our two measures of heat tolerance did not support the idea.

Can our measure of heat-stimulated protein synthesis, that is not taken during the first 30 mins of heat stress – the time during which the adult succumbs and is immobilised in a tolerance test – tell us anything about the processes that affect heat knockdown time? Our measure of protein synthesis is that which occurred during the 1 hour of recovery that followed a mild 1 hour 37 °C heat stimulus. Considerable *in vitro* and *in vivo* research on this type of exposure of *D. melanogaster* or their cells to heat stress provides pertinent understanding of the underlying cellular heat stress response. While the RNA expression of many hundreds of genes are up- or down-regulated within this two hour period (Kültz 2005; Sørensen *et al.* 2005) the level of total protein synthesised in the recovery hour, as visualised by radio-labelled protein on one- and two-dimensional electrophoretic gels, can be partitioned into that due to the conserved Hsps and that due to synthesis of normal cellular proteins (Storti *et al.* 1980; DiDomenico *et al.* 1982b; Stephanou *et al.* 1983) – the functional mRNAs of these 25 °C proteins having been preserved

during the mild stress (Yost *et al.* 1990). In general, immediately after the return to 25 °C cells continue to produce Hsps, with production of 25 °C proteins re-occurring slowly (Yost *et al.* 1990). Gel labelling intensity suggests that a major part of the recovery-hour synthesis is of Hsps, mostly Hsp70. Therefore this measure could reflect either line differences in the capacity to synthesise Hsps or line differences in the capacity to shutdown 25 °C proteins, two processes that may not be independent (DiDomenico *et al.* 1982a).

In general, faster production of Hsps has been associated with increased thermal tolerance (Parsell and Lindquist 1993). Since a major component of our heat-stimulated rate measurement can be attributed to synthesis of Hsps, and this rate only related negatively to knockdown tolerance, our data do not support the idea that faster production of Hsps in general increases heat tolerance. In fact this result is consistent with a recent finding that variation among lines of adult heat knockdown tolerance did not relate to line variation in their rate of Hsp70 production (Jensen *et al.* 2010). However, since the different Hsps have individual tissue and timing profiles of expression (Lindquist 1980; Palter *et al.* 1986) our results do not exclude the possibility that faster synthesis of a specific Hsp might not directly influence adult knockdown time.

While numerous explanations for a negative association are possible two possibilities can be suggested. If slower production of heat-shock proteins is responsible for higher tolerance the heat-inducible Hsp90 could be implicated. Hsp90 is constitutively present in all cells and binds with and stabilises a large number of client, cell-signal proteins (Chiosis *et al.* 2013). If, early in the response to heat, reduced levels of Hsp90 were to occur this could potentially increase heat knockdown time for two reasons. First, Hsp90 is a negative regulator of the cellular heat-shock response (Zou *et al.* 1998; Bharadwaj *et al.* 1999) and lower levels of Hsp90 would be expected to enhance the response, and second Hsp90 contributes directly to pausing the expression of many stress response genes (Sawarkar *et al.* 2012), many of which help protect cells from heat damage, so lower levels could lead to earlier activation of these genes. Note also that Hsp90 has been postulated to down regulate global protein synthesis during stress (Pal 1998). Alternatively,

if faster shutdown in synthesis of 25 °C proteins is responsible for the negative association this could, early in the heat stress, result in less heat-denatured and aggregated proteins that might otherwise interfere with normal cell physiology and decrease knockdown time. If curtailing synthesis of 25 °C proteins proved to be a key factor, investigating the role of the heat stress RNA gene *hsr-omega* that is up-regulated by heat shock in concert with the Hsps, and by the same heat-shock-factor mechanism, would be worthwhile. *Hsr-omega* helps to down-regulate general protein synthesis (Johnson *et al.* 2011) and its expression and genotype variation have been associated with adult heat knockdown time in this species (McKechnie *et al.* 1998). These speculative ideas could be considered in future research. For example, it would be of interest to look for associations of knockdown tolerance specifically with, a) rates of production of individual Hsps, and *hsr-omega* transcripts, during the first 30 min of heat stimulus (as is used to measure knockdown time), and b) rates of shutdown of normal 25 °C proteins in this 30 min period, and compare these with heat knockdown time across a set of lines similar to those used in the current study. Both specific Hsp synthesis and 25 °C protein synthesis could be quantified from radio-labelled gels in the same experiment using established techniques (Storti *et al.* 1980; Stephanou *et al.* 1983). Of course the negative association might be indirect and some common factor that influences both knockdown time and heat-stimulated protein synthesis may be responsible for the association.

In summary, we have used a fairly course measurement of total protein synthesis to see if it relates to levels of heat knockdown time measured in adult female *D. melanogaster*. While the evidence is only correlative it re-affirms, since it largely repeats two similar previous observations, the significance in this species of a negative relationship between levels of heat-stimulated total protein synthesis and heat tolerance. We discuss possible mechanisms that might be responsible for this negative association, in terms of components of the cellular heat stress response, and we suggest a future experiment to narrow down possible explanations. We found no evidence for a positive association between rates of general protein synthesis following heat stress and thermal tolerance, although this result does not exclude the possibility that up-

regulation of any particular heat stress gene might be directly and positively related to knockdown tolerance. Our data clearly do not support the idea that the reported clinal variation in protein synthesis in this species contributes to the latitudinal clines in thermal tolerance.

3.6 Acknowledgements

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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Design, research, analysis, manuscript preparation	60%

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	Extent of contribution (%) for student co-authors only
Travis Johnson	Design, research	10%
Louise Jensen	Research	5%
Carla Sgrò	Analysis, manuscript preparation	10%
Stephen McKechnie	Design, analysis, manuscript preparation	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**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

CHAPTER 4

Hsp90 levels in natural populations of *Drosophila melanogaster* cline with latitude and associate with adaptive heat tolerance variation

4.1 Abstract

Heat stress affects almost every organism and it is becoming increasingly important to understand the adaptive and physiological processes underlying a species' tolerance to heat. In all species Heat shock protein 90 (Hsp90) is an important and abundant cellular factor that chaperones numerous effector proteins in both unstressed and heat-stressed cells. Despite this, the extent to which variation in expression of the *hsp90* gene contributes to naturally occurring variation in thermal tolerance within and between populations remains unknown. Here we characterise natural variation in adult *D. melanogaster hsp90* transcript and Hsp90 protein levels (*hsp83* and Hsp83, respectively, in *Drosophila*) in populations from diverse climatic regions along the eastern Australian coast, where heat tolerance is higher in the tropical low-latitude populations. We first examine three independent sets of isofemale lines for a link between *hsp90* and heat tolerance, finding negative associations between *hsp90* transcript level and adult heat knockdown tolerance. We also find positive latitudinal clines in non-heat-stressed levels of both *hsp90* transcript and Hsp90 protein along the Australian eastern coast – the more heat tolerant tropical populations having less *hsp90* transcript and protein than temperate populations. Since Hsp90 forms complexes with many cell-signal proteins its clinal pattern has potential to influence numerous traits that cline with latitude, including the heat tolerance cline, and thus to help facilitate climatic adaptation. In particular, since Hsp90 is an established negative regulator of the cellular heat shock response, our data suggest that basal tissue level of Hsp90 in *Drosophila* is an important heritable factor contributing to variation in thermal adaptation across diverse climatic regions.

4.2 Introduction

Global environmental change, particularly global warming, is an ongoing threat to the continued well-being of numerous animal and plant species, many of which face extinction unless they can find suitable new habitats or adapt to the increased thermal stress (Somero 2010; Shaw and Etterson 2012). Daily and seasonal heat stress decreases the chances of reproductive success in many species (see for example Kipp 2007; Snider *et al.* 2009; Zizzari and Ellers 2011) and it is important to understand what limits a species' ability to adapt to increasing temperature stress. This has motivated considerable research that has focussed on elucidating the underlying physiology of natural variation in heat tolerance, both within and between species (see for example Fangue *et al.* 2006; Chown and Terblanche 2007; Cossani and Reynolds 2012). *Drosophila* is ideal for studying adaptive natural variation in heat tolerance since extensive variation in thermal tolerance occurs between closely related species (Garbuz *et al.* 2003; Kellett *et al.* 2005), and between populations of a single species from thermally diverse habitats (Zatsepina *et al.* 2001; Sisodia and Singh 2010). A prime example is the latitudinal clines in thermal tolerance of *D. melanogaster* on different continents (Guerra *et al.* 1997; Fallis *et al.* 2011) and particularly along the eastern coast of Australia (Hoffmann *et al.* 2002; Sgrò *et al.* 2010; Cockerell *et al.* 2014). Temperature is thought to be the major environmental variable imposing spatial selection on traits and their underlying genes along latitudinal gradients (Endler 1977) – we know for example that average temperatures increase with decreasing latitude along the Australian eastern coast (Hoffmann 2010). Persistent latitudinal patterns of a trait like heat tolerance strongly suggest that the trait is adaptive. Such clines can be used to investigate how adaptation occurs – to establish the links between fitness, thermal physiology, cellular mechanisms, and candidate genes.

While whole genomic studies reveal hundreds of genes that are involved in responding to heat stress in *Drosophila* (Birch-Machin *et al.* 2005; Sørensen *et al.* 2005) we are only beginning to understand the cellular and physiological processes that vary in natural populations and underpin

adaptive heat tolerance variation. Tolerance to heat can be measured in different ways, some of which are likely to be more ecologically relevant than others (Terblanche *et al.* 2011). Whereas adult heat knockdown time, measured either before or after a period of rapid heat acclimation, is used to demonstrate adaptive geographical variation (Sgrò *et al.* 2010) other heat-related fitness components such as reproductive success following heat stress may be more significant from an ecological and evolutionary perspective. Nonetheless, whichever fitness measurements are used to assess adaptive heat tolerance variation, components of the highly conserved cellular heat stress response are likely to be involved in the underlying mechanism.

We currently have considerable knowledge about the cellular heat stress response - it is the first line of defence against heat stress and involves the rapid regulation of a diverse set of heat shock response genes following heat stress (Kültz 2005). The response is primarily regulated by Heat shock factor (Hsf in *Drosophila* or Hsf1 in most eukaryotes), a protein that forms trimers at increased temperatures with consequential activation of transcription of a conserved set of stress response genes (Akerfelt *et al.* 2010). Among these, and well characterised, are the genes encoding the Heat shock proteins (Hsps) that are swiftly up-regulated and play a major role in providing molecular chaperones for the cell. Their main function is to protect normal cellular proteins thus minimising their denaturation and aggregation – the different Hsps being tissue- and organelle-specific and chaperoning different client proteins (Feder and Hofmann 1999; Tutar and Tutar 2010). In parallel, the synthesis of normal cellular heat-sensitive proteins is curtailed, conserving energy and freeing up the translational machinery for faster synthesis of protective proteins. In *D. melanogaster* the primary response genes comprise two members of the *hsp70* family (*hsp70* and *hsp68)*, *hsp90*, a set of four small *hsp* genes, and *hsr-omega* (Pardue *et al.* 1992; Parsell and Lindquist 1993). The main RNA product of *hsr-omega* helps shut down synthesis of normal cellular house-keeping proteins following heat stress (Johnson *et al.* 2011). These components of the cellular heat shock response provide key starting points to identify cellular mechanisms and genes that underpin natural heritable variation in thermal tolerance.

In *Drosophila* the latitudinal cline in thermal tolerance along the Australian eastern coast coincides with latitudinal clines in frequency of *hsp70,* small *hsp23* and *hsr-omega* (discussed in Hoffmann and Weeks 2007) suggesting a causal connection for these components. Expression of one small heat shock protein in *Drosophila* has also been associated with micro-geographic variation in heat tolerance (Carmel *et al.* 2011). However, natural variation in expression of the predominant protective protein, Hsp70, appears not to relate to adult thermal tolerance variation (Jensen *et al.* 2010). One *hsp* gene that has not been thoroughly investigated in the context of thermal tolerance is *hsp90*. Unlike most other heat shock proteins Hsp90 is ubiquitous and abundant in unstressed cells (Lai *et al.* 1984; Chiosis *et al.* 2013), as well as being further synthesised following heat stress. It plays a major role in complexing with, stabilising, and/or activation of an estimated 5% of all cellular proteins, so called Hsp90 client proteins (Chiosis *et al.* 2013). These client proteins are essential for many cell signal transduction pathways that are required for normal cellular functions (Csermely *et al.* 1998; Zuehlke and Johnson 2009), particularly for the stressed cell, and it has the potential to influence many phenotypic and physiological traits (Rutherford *et al.* 2007; Zhao and Houry 2007; Sawarkar *et al.* 2012). *Hsp90* has been extensively studied for its role in phenotypic canalisation - the buffering of phenotypic trait variation (Rutherford and Lindquist 1998; Debat *et al.* 2006; Milton *et al.* 2006; Rutherford *et al.* 2007).

In a few organisms variations in Hsp90 protein, or the *hsp90* gene, have been examined for effects on traits related to fitness following thermal stress. For example, seasonal and heat- and cold-stress induced changes in *hsp90* transcript level have been found in the onion maggot, *Delia antique* (Chen *et al.* 2005), and *Arabidopsis* plants treated with a *hsp90* inhibitor survived temperatures lethal to control plants, suggesting a negative association between *hsp90* and heat tolerance (McLellan *et al.* 2007). One rare natural *hsp90* variant in *D. melanogaster* had no detectable effect on heat tolerance (Rako *et al.* 2007). However Chen and Wagner (2012) found that naturally occurring mutations in *hsp90* in *Drosophila* reduced *hsp90* expression, reduced fitness components such as larval competition, fecundity and longevity and also reduced heat

resistance. Thus, while some interesting relationships between *hsps* and thermal tolerance have been suggested, no systematic effort to relate *hsp90* expression to natural variation in thermal tolerance of *Drosophila* has been reported.

In this report we characterise *hsp90* transcripts and protein levels in adult *D. melanogaster* in three independent sets of laboratory lines and we look for associations with adult heat knockdown tolerance. Since we find consistent negative relationships we examine the possibility that *hsp90* variation may play a role in adaptive heat tolerance that clines with latitude along the eastern coast of Australia. We found that *hsp90* expression does change with latitude, consistent with the possibility that it contributes to the tolerance cline, and we discuss the implications of the *hsp90* cline for its effects on other traits and on initiation of the heat shock response.

4.3 Materials and methods

4.3.1 Collection and maintenance of lines and populations

Coffs Harbour lines: Twenty isofemale lines of *D. melanogaster* were established from field inseminated females collected at Coffs Harbour (30.16 °S) NSW, mid-latitude in the thermal tolerance cline on the east coast of Australia in May 2005, maintained at 25 °C on agar-sugar-yeast-oatmeal medium with a 12:12 h light/dark cycle in the laboratory at The Bio 21 Institute at the University of Melbourne where the tolerance testing and *hsp90* transcript levels were determined.

Bonville lines: Forty lines of *D. melanogaster* that were selected from a larger set were established as described in Jensen *et al.* (2010: the *Study 2* lines). These lines were established from field-inseminated females collected from Bonville (latitude 30.22 °S; 11.5 km south of Coffs Harbour) in 2006 and were maintained at Monash University on a potato-yeast-dextrose-agar medium at 25 °C with a 12:12 h light/dark cycle.

Hybrid lines: From the eastern Australian latitudinal transect three southern populations (latitudes 43.15 °S, 37.77 °S and 36.92 °S) and three northern populations (19.97 °S, 18.20 °S, 17.52 °S) were used to establish a hybrid population that contained gene combinations from the entire latitudinal transect. After mass rearing in the laboratory for twelve generations, to facilitate genome mixing (thus disrupting gene combinations from the cline ends), and to minimise latitude specific linkage disequilibrium, we established from this population a set of 40 family lines that are used in the current study (details of the derivation are described in Cockerell *et al.* 2013). Lines were maintained at Monash University in large numbers (duplicate culture bottles each containing 80 ml medium - flies being mixed each generation and approximately 300 flies contributing to egg production for four hours per bottle, per generation) on a potato-yeast-dextrose-agar medium at 25 °C with a 12:12 h light/dark cycle.

Transect populations: Populations were sampled in February 2009 from eight points along a latitudinal gradient on the Australian east coast (Northern Tasmania 41.24 °S, Melbourne 37.78 °S, Gosford 33.29 °S, Coffs Harbour 30.38 °S, Maryborough 25.54 °S, Rockhampton 23.45 °S, Bowen 19.98 °S, Innisfail 17.53 °S). Ten isofemale lines were first established for each location. Following three generations of culture in the laboratory two mass bred populations were generated for each location by combination of 20 males and virgin females from each of five isofemale lines from that location. For all locations each of the two genetically separate populations was immediately reared to large numbers (as described above) and treated as one of two replicate location samples. These populations were established and tested at Monash University and maintained on a potato-yeast-dextrose-agar medium at 25 °C with a 12:12 h light/dark cycle.

4.3.2 Heat tolerance assays

Coffs Harbour lines: Prior to testing densities were controlled by allowing parental flies to oviposit for 12 h on spoons with food media. Twenty five larvae or eggs were then counted and distributed to each vial for development and rearing. Adult basal and hardened heat knockdown tolerance was assessed for 10 day old mated females reared under constant 25 °C conditions. Ten females per line per treatment were set up individually in glass vials, sealed with a plastic cap, placed at random into racks, and submerged in a recirculating water bath at 38.5 °C. The time taken for the flies to be completely immobilised (knockdown time) was recorded. Multiple runs in the water bath were necessary and any effect of run was adjusted for as described below. Hardening treatment involved exposure of the females to 35 °C for 1 h followed by recovery for 1 h at 25 °C before submersion at 38.5 °C. Testing occurred 25 generations after lines were collected.

Bonville lines: Adult basal and hardened heat knockdown tolerances were assessed for 5-7 day old females. Tolerance testing was performed at F_3 after the forty lines were established. Basal and hardened heat knockdown times were measured at different times by placing six females per line together in 5-ml glass vials and exposing acutely to 39 °C by immersion of multiple-vial racks in a preheated water bath, all lines being tested at the same time. The time for individual flies to be knocked down and immobilised was averaged for each vial and recorded. The hardening treatment involved exposing flies in empty 42 ml vials flies to 37 °C for 1 h submerged in a water bath, with 6 h recovery at 25 °C on fly food immediately prior to testing.

Hybrid lines: Prior to testing densities were controlled for three generations by having a short egg-laying period, and to generate the adults for testing, 50 eggs were placed into each rearing vial after having the parents lay eggs onto small spoons of medium for collection and counting. Adult basal and hardened heat knockdown tolerance was assessed for 5-7 day old females. Tolerance testing was performed at F_7 following line establishment. Basal and hardened heat

knockdown times were measured by placing flies individually in 5-ml glass vials and exposing acutely to 39 °C by immersion of multiple-vial racks in a preheated water bath. The time for individual flies to be knocked down and immobilised was recorded. The hardening involved exposure to 37 °C for 1 h, with 6 h recovery at 25 °C prior to testing. For each line 24 females (12 hardened and 12 basal) were tested randomly over 10 runs over two days, with all runs containing equal numbers of each treatment.

Transect populations: Densities were controlled as described above for the Hybrid lines. Adult basal and hardened heat knockdown tolerance was assessed for 5-7 day old females using the same protocol as for the Hybrid lines, at F_8 after establishing the duplicate populations in the laboratory. For each population, 30 females were tested per treatment (hardened and basal) randomly over 10 runs over two days, with all runs containing equal numbers of flies from each population and each treatment.

4.3.3 Quantification of hsp90 *transcript*

Coffs Harbour lines: real-time quantitative PCR was used to assess *hsp90* transcript levels in non-heat treated and heat stimulated females. Testing occurred 25 generations after collection, with rearing density controlled as described above for tolerance testing of the Coffs Harbour lines. Three groups of 15 adult females per population, per heat treatment, were used to extract RNA using RNeasy (QIAGEN) with on-column digestion. For the heat stimulus females were given a 35 °C heat stimulus for 1 h, followed by recovery at 25 °C for 1 h on food before being snap frozen in liquid nitrogen and stored at -80 °C until assayed. Flies were homogenised according to the manufacturer's protocol using rotor-stator homogenisation. cDNA synthesis was completed using First-Strand cDNA synthesis kit (Amersham Biosciences) with the first strand $pd(N)_6$ primer provided by the kit. After synthesis the cDNA was diluted 1:250 in water and stored at -20 °C. Reactions were set up on ice using 7.5 µl water, 12.5 µl Stratagene Brilliant® SYBR® Green QPCR Master Mix, 0.5 µl of each primer (10 µM; *hsp90:* F-5'-TGT GAA TTT

CCA ATT CTA TAC AAA GCA AAG, R-5'-GCT TCT TCT GGC ATC TTG TAT GTA TGT T, and for *actin 5C:* F-5'-CCG TCA CCA GGG TGT GAT GGT, R-5'-CAG TTG GTC ACG ATA CCG TGC TCA) and 4 µl of cDNA, and were briefly centrifuged before being analysed on a Stratagene MX3005P platform. Each sample was run in duplicate and all lines from each biological replicate were processed together in one run. The amount of target gene expression was estimated using a standard curve of four points in duplicates prepared from dilutions of a pool of the sample cDNAs. *Hsp90* transcript measures were internally normalized to *actin 5C* levels. To compare transcripts between samples, levels were established by obtaining cycle threshold (Ct) differences between *actin 5C* and *hsp90* values for each RNA extract and then converting to a fold difference value (Schmittgen and Livak 2008).

Bonville lines: *hsp90* transcript levels were quantified for heat-stimulated and non- treated females using the real time RT-PCR protocol described by Collinge *et al.* (2008) with slight modification. Transcript level determinations were carried out at F_{10}. For the heat treatment groups of 20 adult females were given a 37 °C 1 h heat stimulus, followed by 6 h recovery at 25 °C. RNA was extracted from one group of 20 adult females per line per treatment using TriSure reagent (Bioline, Alexandria, NSW, Australia) as per manufacturer's instructions, followed by DNaseI treatment (New England Biolabs) for 40 min at 37 °C prior to cDNA conversion. Complementary DNA was synthesised using 2 µg of total RNA primed with 200 ng of random primers (Invitrogen, Mt Waverley, VIC, Australia) and using BioScript reverse transcriptase (Bioline), according to manufactures instructions. Real-time PCR was performed in the Rotor-Gene 6000 (Corbett Lifescience, Mortlake, NSW, Australia). Measures were internally normalized to levels of *cyclin K* chosen because in microarray studies it showed particularly low variance across heat treatments (Sørensen *et al.* 2005). Duplicate PCR reactions using transcript-specific primers (400 nM) for *hsp90* (F-5'-CAT ACA AGA TGC CAG AAG AAG C, R-5'-TGG GGT CAG TAA GGG ACT CA) and for *cyclin K* (F-5'-GAG CAT CCT TAC ACC TTT CTC CT, R-5'-TAA TCT CCG GCT CCC ACT G) were completed for each cDNA batch using the Sensimix DNA kit (Quantace, Alexandria, NSW, Australia). Amplification conditions were

95 °C 10 min followed by 50 cycles of 95 °C (10 s), 58 °C (15 s) and 70 °C (5 s) with fluorescence data acquisition at the completion of the 70 °C step every cycle. Following cycling, product melt curves were generated by raising temperature from 69 to 90 °C in 1 °C increments. Primer pairs were previously validated across a range of cDNA concentrations with efficiencies found to be within the acceptable ranges. For transcript level analysis, Ct differences between *cyclin K* and *hsp90* for each RNA extract were converted to fold differences.

Hybrid lines and Transect populations: *hsp90* transcript levels were quantified both before and after heat stimulus (37 °C 1 h followed by 6 h recovery at 25 °C) using qRT-PCR. RNA extraction and conversion to cDNA was as described above for the Bonville lines. Transcript determination was performed on F_7 individuals following line establishment for the Hybrid lines, and F_5 following establishing the 16 Transect populations. For each line and each population, three replicate RNA extractions of 20 adult females per treatment were obtained. RT-PCR was performed in the LightCycler® 480 (Roche) system as per Lee *et al.* (2011a) with modifications as follows: PCR reactions were set up with 50% universal buffer (manufacturer's instructions, LightCycler® 480 High Resolution Melting Master, Roche), 10 µg cDNA (1% of reaction) and 400 nM (0.4% of reaction each) *hsp90*-specific primers (as for the Bonville lines). Four technical replicates were performed per biological replicate. To compare transcripts between samples, *hsp90* transcript measures were internally normalized to *cyclin K* levels by obtaining Ct differences between *cyclin K* (primers as above) and *hsp90* values for each RNA extract and then converting to a fold difference value.

4.3.4 Hsp90 protein level in Bonville lines and Transect populations

Hsp90 protein levels were quantified from band intensities on Western blots for both heat-stimulated females and non-heat stimulated females. For the heat stimulation groups of ten adult females were transferred to plastic-capped 40 ml vials containing 2 ml of food medium and

given a 37 °C heat shock, submerged in a recirculating water bath for 1 h, followed by 6 h recovery at 25 °C on food in vials sealed with a foam plug. At the end of the recovery time flies were snap frozen in liquid nitrogen and stored at -80 °C until assayed. Protein level determinations were carried out at F_{10} for the Bonville lines and at F_8 for the Transect populations. Whole-fly protein extractions were carried out as described for study 2 in (Jensen *et al.* 2010). Protein concentrations were assessed (BCA Protein Assay Kit; Pierce Biochemicals) and samples were diluted to 1800 μg/ml. Proteins were separated via SDS-polyacrylamide gel electrophoresis (PAGE). Protein expression was measured using monoclonal primary antibodies (Hsp90, #4874 Cell Signalling Technology and α-Tubulin, T6074 Sigma-Aldrich) diluted to a concentration of 1/1000 (Hsp90) or 1/100,000 (α-Tubulin). Secondary antibodies (anti-rabbit IgG, #7074, Cell Signaling Technology, and anti-mouse IgG, #HAF007, R&D Systems, for Hsp90 and α-Tubulin detection, respectively) were diluted at 1/2000. Membranes were exposed to x-ray film (Amersham-Pharmacia). Western blots were quantified using AlphaImager and ImageQuant TL version 2005. A large protein extraction of adult flies from a mass bred Coffs Harbour population was stored in aliquots at -80 °C and used as standards to compare across Western blots. These were treated identically to samples and loaded in duplicate onto every gel. Standardisation of the sample involved dividing the blot band intensity by the α-Tubulin value for that sample, and then dividing by the average image value of the standard from that Western blot. Gel effects were also taken into account by multiplying each standardised sample by overall mean / gel mean (Jensen *et al.* 2010).

4.3.5 Data analysis

Within sets of lines or populations time data from heat tolerance tests were corrected for any effects of run by multiplying each knockdown time by grand mean/run mean (Sgrò *et al.* 2010). To test for differences in *hsp90* transcript and protein levels between populations, and interactions between treatment and population, two-way analyses of variance were performed. Linear regression analyses were performed to test for relationships between the traits examined, as well as relationship with latitude. In the case of multiple comparisons Bonferroni corrections were applied. Pearson correlation coefficients were used to test for associations between variables measured in the same sets of lines or populations. Analyses were performed using SPSS 18.0 for windows and JMP 6.0.0 (SAS Institute Inc.).

4.4 Results

4.4.1 Heat knockdown time

We assessed heat knockdown time before and after heat hardening in four sets of lines or populations, the Coffs Harbour lines, the Bonville lines, the Hybrid lines and the set of Transect populations. As would be expected after a mild heat-hardening stress (Sgrò *et al.* 2010), strong increases in knockdown time were detected in all these *D. melanogaster* lines and populations (Table 4.1). Within each set there were differences among lines or populations for knockdown time, and for all but the Hybrid line set there were significant differences among lines or populations in the extent of the hardening response (see the interactions in Table 4.1).

4.4.2 Genetic variation in hsp90 *expression*

The four sets of lines or populations were assessed for *hsp90 t*ranscript or Hsp90 protein levels at different times (and for the transcript, in different laboratories) so that variations in levels among sets cannot be meaningfully compared. Also, within each of the sets of lines or populations little

association was detected between basal and heat-stimulated levels of *hsp90* transcript, between basal and heat-stimulated levels of Hsp90 protein, or between levels of transcript and levels of protein. The only significant associations were in the Bonville lines – a positive association occurred between basal and heat-stimulated levels of *hsp90* transcript (Table 4.2), and a negative association was detected between basal levels of transcript and heat-stimulated levels of Hsp90 protein.

Among the Coffs Harbour lines significant variation in *hsp90* transcript level was not detected. However, heat stimulus treatment significantly increased average levels of *hsp90* transcript by 5.1 fold above the basal levels (Table 4.3). The absence of an interaction effect suggests that the Coffs Harbour lines did not differ in the extent of increase in transcript following heat stimulus. Hsp90 protein production was not examined in the Coffs Harbour lines.

Among the Bonville lines, single RNA extracts per line precluded testing for line effects on *hsp90* transcript levels. However in these lines the heat stimulus significantly increased average *hsp90* transcript levels, by 2.9 fold above the basal level (Table 4.3; $F_{(1,78)} = 393.72$, $p < 0.0001$). No significant variation in Hsp90 protein level among the Bonville lines was detected. However, the average Hsp90 protein level was, significantly, 21% less in heat-stimulated flies (Table 4.3). While the extent of change in Hsp90 protein level following heat stimulus appeared different among the Bonville lines there was no significant heterogeneity - indicated by the non-significant interaction term in Table 4.3.

No significant variation in *hsp90* transcript level was evident among the Hybrid lines. The heat stimulus treatment significantly increased average *hsp90* transcript, by 1.7 fold above basal levels in these lines (Table 4.3). Significant line by treatment interaction indicated that the Hybrid lines responded differently to heat-stimulus in the extent to which *hsp90* transcript level was increased (Table 4.3). Hsp90 protein production was not examined in the Hybrid lines.

Among the Transect populations significant variation in *hsp90* transcript level, but not protein level, was detected among populations (Table 4.3). The heat stimulus treatment significantly increased average *hsp90* transcript by 1.6 fold above basal levels, and increased Hsp90 protein by 1.2 fold above basal levels. Levels of *hsp90* transcript in the Transect populations displayed significant population by treatment interaction suggesting that the populations responded differently to heat stimulus in elevating transcript levels, a result not evident for Hsp90 protein levels in these populations (Table 4.3).

Table 4.1: Two-way analysis of variance (Anova) testing for population differences for basal and hardened heat knockdown tolerance in populations of *D. melanogaster*.

Data set (lines/populations)	Source	df	SS	*F*	*p*-value
Bonville lines	Line	38	9960.83	9.17	<0.0001
	Treatment	1	1398.88	48.93	<0.0001
	Line x treatment	38	2550.66	2.35	<0.0001
	Error	1101	31476.48		
Hybrid lines	Line	40	7602.56	4.17	<0.0001
	Treatment	1	7225.14	158.43	<0.0001
	Line x treatment	37	1825.98	1.08	0.3417
	Error	819	37351.00		
Coffs lines	Line	19	4528.44	1.80	0.0218
	Treatment	1	20007.70	150.90	<0.0001
	Line x treatment	19	4081.32	1.62	0.0492
	Error	347	46007.34		
Clinal populations	Population	15	4143.35	6.82	<0.0001
	Treatment	1	3635.87	89.76	<0.0001
	Population x treatment	15	1099.99	1.81	0.0292
	Error	893	36172.82		

Table 4.2: Associations between basal and heat-shocked *hsp90* transcript, basal and heat-shocked protein and between *hsp90* transcript and protein. Pearson's correlation coefficients (*r*) are displayed with p-values (two-tailed tests of significance).

Lines/Populations	***hsp90***	***hsp90***	**r**	***p***
Basal vs heat-shocked transcript				
Coffs	Basal transcript	Heat-shocked transcript	-0.062	.796
Bonville	Basal transcript	Heat-shocked transcript	0.516	.001
North/South Hybrid	Basal transcript	Heat-shocked transcript	0.339	.040
Cline	Basal transcript	Heat-shocked transcript	-0.247	.555
Basal vs heat-shocked protein				
Bonville	Basal protein	Heat-shocked protein	0.128	.500
Cline	Basal protein	Heat-shocked protein	-0.544	.163
Transcript vs protein				
Bonville	Basal transcript	Basal protein	0.043	.550
	Basal transcript	Heat-shocked protein	-0.339	.002
	Heat-shocked transcript	Basal protein	-0.160	.322
	Heat-shocked transcript	Heat-shocked protein	-0.339	.122
Cline	Basal transcript	Basal protein	0.632	.093
	Basal transcript	Heat-shocked protein	-0.427	.292
	Heat-shocked transcript	Basal protein	0.096	.822
	Heat-shocked transcript	Heat-shocked protein	0.509	.198

Table 4.3: Two-way analysis of variance (Anova) testing for population differences for basal and heat-shocked *hsp90* transcript and protein levels in populations of *D. melanogaster*.

Trait	Data set (lines/populations)	Source	df	SS	*F*	*p*-value
hsp90 transcript	Coffs lines	Line	19	0.87	1.38	0.1601
		Treatment	1	86.56	2602.71	<0.0001
		Line x treatment	19	0.99	1.57	0.0870
		Error	78	2.59		
	Hybrid lines	Line	39	2734.11	3.34	<0.0001
		Treatment	1	4991.63	237.63	<0.0001
		Line x treatment	38	1672.35	2.10	0.0011
		Error	134	2814.77		
	Clinal populations	Population	7	2968.46	3.12	0.0059
		Treatment	1	2188.40	16.10	0.0001
		Population x treatment	7	4801.60	5.05	0.0001
		Error	77	10463.71		
Hsp90 protein	Bonville lines	Line	38	10.82	1.55	0.0407
		Treatment	1	2.20	11.98	0.0008
		Line x treatment	38	7.35	1.05	0.4054
		Error	112	20.57		
	Clinal populations	Population	15	0.27	0.74	0.7387
		Treatment	1	0.32	13.23	0.0004
		Population x treatment	15	0.46	1.28	0.2286
		Error	107	2.57		

4.4.3 Associations between heat tolerance and hsp90 *expression*

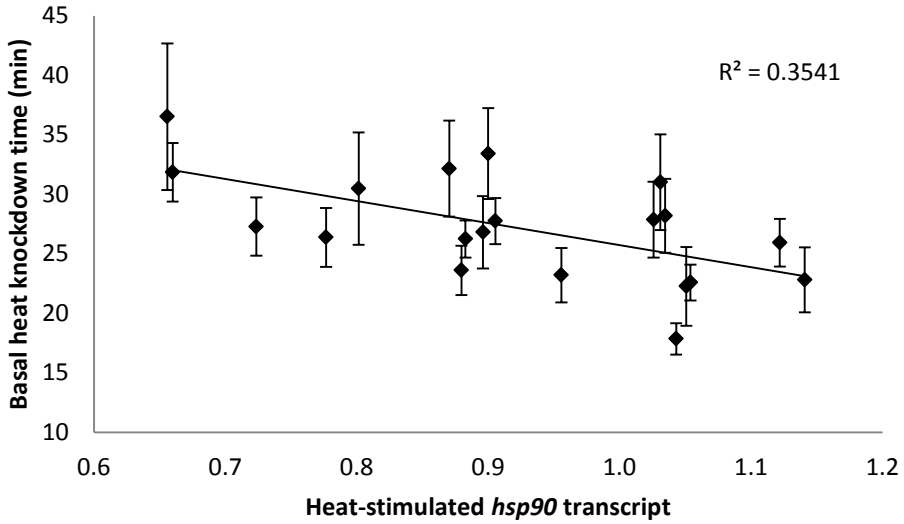
In each of the four sets of lines or populations we characterised basal and heat-hardened adult heat knockdown time and basal and heat-stimulated levels of *hsp90* transcript. We also characterised basal and heat-stimulated levels of Hsp90 protein, but only in the Bonville lines and Transect populations. Thus there were 24 opportunities to look within sets for associations between expression of the *hsp90* gene and heat tolerance. Among the Coffs Harbour lines a negative relationship was detected between heat-stimulated levels of *hsp90* transcript and basal heat knockdown time (Fig. 4.1 Table 4.4). Among the Bonville lines basal *hsp90* transcript level and basal heat knockdown time were negatively related (Fig. 4.1, Table 4.4). Also, in the Bonville lines basal Hsp90 protein abundance was also negatively related to hardened heat knockdown time (Fig. 4.2, Table 4.4). Among the Hybrid lines basal *hsp90* transcript level and basal heat knockdown time were negatively related (Fig. 4.3, Table 4.4). The Hybrid line data also suggested a negative relationship between basal *hsp90* transcript and hardened heat knockdown time, however this result was marginally non-significant (Table 4.4). Heat stimulated *hsp90* transcript levels and basal heat knockdown times were also negatively related in the set of Hybrid lines (Fig. 4.3, Table 4.4).

Among the Transect populations a negative relationship was found between basal *hsp90* transcript level and basal heat tolerance, as was indicated for basal Hsp90 protein level and basal heat tolerance (albeit marginally non-significant for the latter relationship, Table 4.4). In contrast, heat-stimulated transcript levels among the Transect populations were positively related, at a borderline level of significance (Table 4.4), to hardened heat tolerance.

Overall, with only the one latter exception, significant negative associations between measures of *hsp90* transcript or Hsp90 protein and heat tolerance were indicated across the three sets of lines and Transect populations. Among the 24 possible assessments of a relationship between one or other measures of expression of the *hsp90* gene and adult heat knockdown tolerance, 17

cases of a negative direction for the line-of-best-fit were detected (Table 4.4), a result that deviates significantly from the 50:50 ratio that would be expected if *hsp90* had no relationship to heat tolerance (χ^2 = 4.17, 1df, $p < 0.05$). Although significance levels were occasionally low, higher levels of *hsp90* expression were more frequently associated with lower knockdown heat tolerance.

(a)



(b)

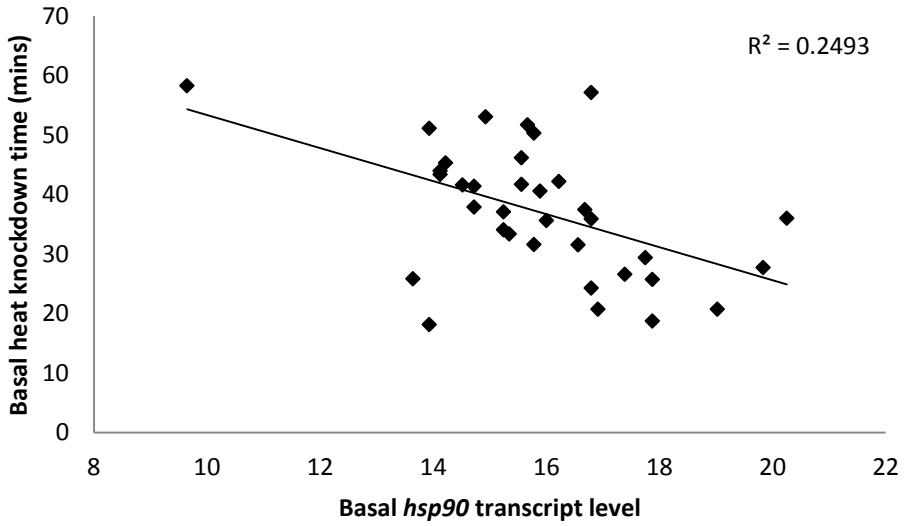


Fig. 4.1 *Hsp90* transcript in *D. melanogaster* and its association with adult heat knockdown tolerance. (a) Heat-stimulated *hsp90* transcript level versus basal knockdown time in the Coffs Harbour lines. Error bars: ± 1 standard error. (b) Basal *hsp90* transcript versus basal heat knockdown tolerance in the Bonville lines.

Table 4.4: Regression analyses for relationships between *hsp90* transcript or protein and heat knockdown in *D. melanogaster* lines/populations. Line/population means are used as data points in the analyses.

Lines/Populations	*hsp90*	Heat knockdown	R^2	b ± SE	*t*	*p*-value
***hsp90* transcript**						
Coffs	Basal	Basal	0.0016	-3.99 ± 23.22	-0.17	0.8656
	Basal	Hardened	0.0087	-663.18 ± 1665.70	-0.40	0.6952
	Heat-shocked	Basal	0.3541	-18.53 ± 5.90	-3.14	0.0056
	Heat-shocked	Hardened	0.0006	56.43 ± 528.21	0.11	0.9161
Bonville	Basal	Basal	0.4993	-2.77 ± 0.84	-3.31	0.0023
	Basal	Hardened	0.0778	-1.29 ± 0.76	-1.69	0.0995
	Heat-shocked	Basal	0.1181	-0.44 ± 0.21	-2.10	0.0433
	Heat-shocked	Hardened	0.0711	-0.29 ± 0.18	-1.61	0.1158
North/South Hybrid	Basal	Basal	0.1252	-0.25 ± 0.11	-2.30	0.0271
	Basal	Hardened	0.0887	-0.24 ± 0.13	-1.85	0.0734
	Heat-shocked	Basal	0.1501	-0.24 ± 0.09	-2.59	0.0135
	Heat-shocked	Hardened	0.0401	-0.15 ± 0.12	-1.23	0.2282
Cline	Basal	Basal	0.8290	-0.17 ± 0.03	-5.39	0.0017
	Basal	Hardened	0.4641	-0.16 ± 0.07	-2.28	0.0628
	Heat-shocked	Basal	0.2287	0.11 ± 0.08	1.33	0.2307
	Heat-shocked	Hardened	0.5366	0.20 ± 0.07	2.64	0.0388
	Basal	Latitude	0.8291	1.12 ± 0.21	5.40	0.0017
	Heat-shocked	Latitude	0.0604	-0.26 ± 0.42	-0.62	0.5575
Hsp90 protein						
Bonville	Basal	Basal	<0.0001	0.02 ± 2.10	0.01	0.9920
	Basal	Hardened	0.1194	-4.16 ± 2.03	-2.05	0.0489
	Heat-shocked	Basal	0.0321	1.62 ± 1.50	1.08	0.2883
	Heat-shocked	Hardened	0.0031	0.52 ± 1.57	0.33	0.7446
Cline	Basal	Basal	0.4610	-20.12 ± 8.89	-2.26	0.0642
	Basal	Hardened	0.3337	-20.882 ± 12.04	-1.73	0.1337
	Heat-shocked	Basal	0.0045	-3.16 ± 19.18	-0.16	0.8746
	Heat-shocked	Hardened	0.2306	27.55 ± 20.55	1.34	0.2284
	Basal	Latitude	0.6187	-0.01 ± 0.00	3.12	0.0206
	Heat-shocked	Latitude	0.0542	-0.0011 ± 0.00	-0.59	0.5791

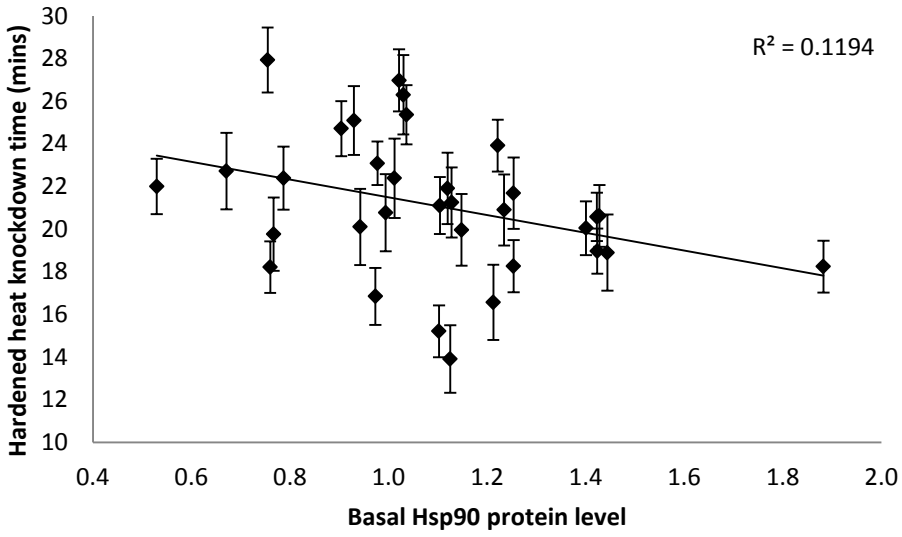
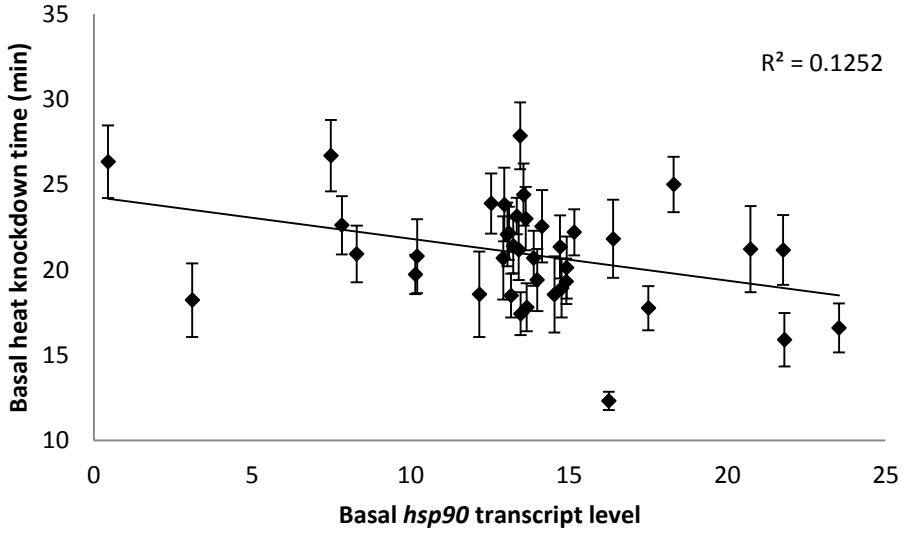


Fig. 4.2 Association between basal Hsp90 protein level and hardened heat knockdown time in Bonville lines of *D. melanogaster*. Error bars: ± 1 standard error.

(a)



(b)

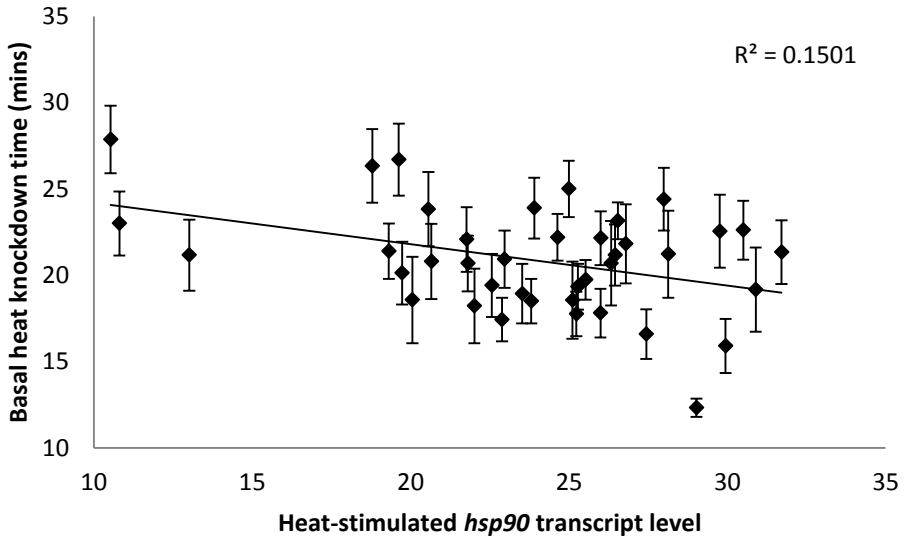
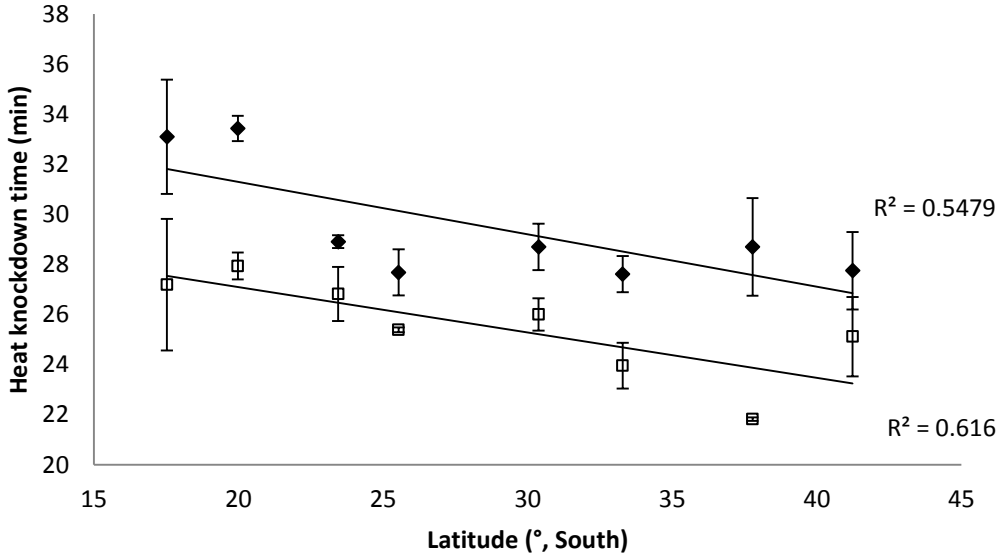


Fig. 4.3 *Hsp90* transcript in *D. melanogaster* from the Hybrid lines and its association with adult knockdown heat tolerance. (a) Basal *hsp90* transcript versus basal knockdown time. (b) Heat-stimulated *hsp90* transcript level versus basal knockdown time. Error bars ± 1 standard error.

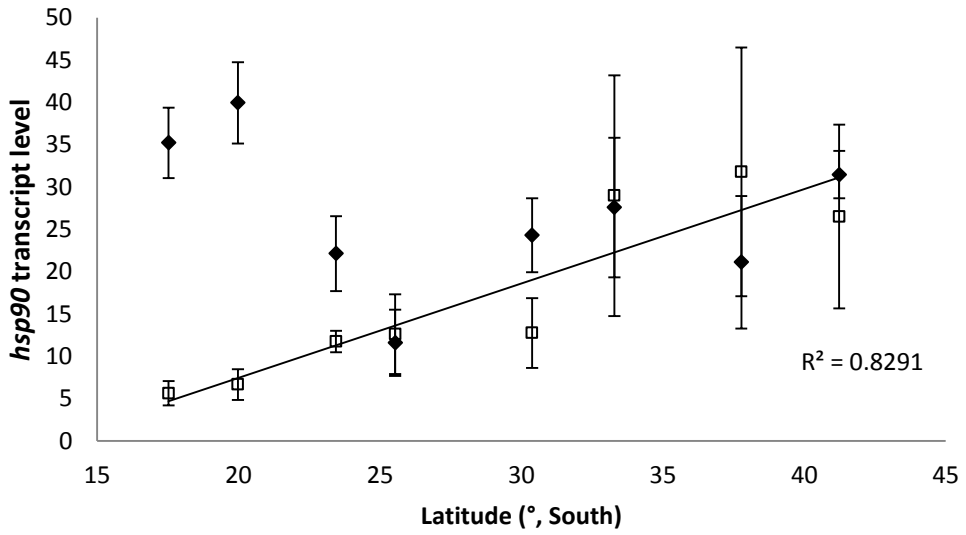
4.4.4 Latitudinal associations

Basal and heat-hardened adult knockdown tolerance, levels of *hsp90* transcript, and levels of Hsp90 protein were examined from populations of *D. melanogaster* collected from eight sites spread over a latitudinal gradient along the Australian east coast. Among these Transect populations we confirmed the negative linear association of latitude with both basal heat tolerance (R^2 = 0.616, t = -0.182, p = 0.021) and hardened heat tolerance (R^2 = 0.548, t = -0.209, p = 0.036; Fig. 4.4) – results consistent with previous reports (Hoffmann *et al.* 2002; Sgrò *et al.* 2010). A significant positive linear cline (latitudinal association) was also observed for basal *hsp90* transcript level (Fig. 4.4, Table 4.5), with low-latitude tropical populations producing a lower constitutive level of *hsp90.* No latitudinal pattern was observed for heat-stimulated levels of *hsp90*. A significant linear clinal pattern in basal Hsp90 protein level was also detected (Fig. 4.4, Table 4.5), however no such pattern emerged for heat-stimulated levels of Hsp90 protein.

(a)


Heat knockdown time (min)
38
36
34
32
30
28
26
24
22
20
15
20
25
30
35
40
45
Latitude (°, South)
R² = 0.5479
R² = 0.616

(b)


hsp90 transcript level
50
45
40
35
30
25
20
15
10
5
0
15
20
25
30
35
40
45
Latitude (°, South)
R² = 0.8291

(c)

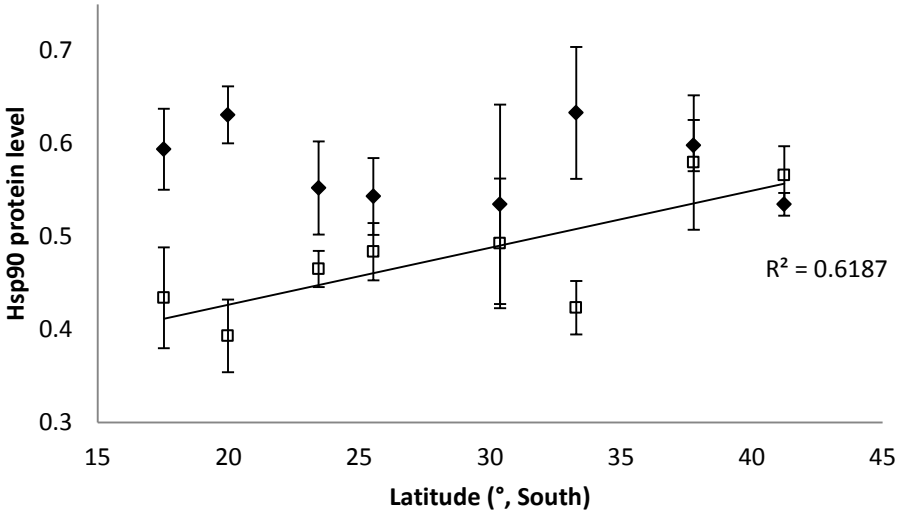


Fig. 4.4 Latitudinal patterns in *D. melanogaster* Transect populations collected from along the east coast of Australia for heat treated levels (filled diamonds) and basal levels (open squares) of, (a) heat tolerance, (b) *hsp90* transcript, and (c) Hsp90 protein. Error bars: ± 1 standard error.

Table 4.5: Regression analyses for relationships between latitude and *hsp90* transcript or protein. Line/population means are used as data points in the analyses.

	***hsp90* (independent)**	**Heat knockdown (dependent)**	R^2	**b ± SE**	***t***	***p*-value**
***hsp90* transcript**						
Cline	Latitude	Basal	0.8291	1.1152 ± 0.207	5.40	0.0017
	Latitude	Heat-shocked	0.0604	-0.2590 ± 0.417	-0.62	0.5575
Hsp90 protein						
Cline	Latitude	Basal	0.6187	-0.0061 ± 0.002	3.12	0.0206
	Latitude	Heat-shocked	0.0542	-0.0011 ± 0.002	-0.59	0.5791

4.5 Discussion

Consistent with previous work on *hsp90* expression transcript level significantly increased after heat treatment in all four sets of lines and populations (Lindquist 1980; Palter *et al.* 1986; Ahmed and Duncan 2004). Hsp90 protein level also increased after heat treatment in the Transect populations. Although significant, the fold increase in Hsp90 protein was only small when compared to that for other heat shock proteins, such as Hsp70 which increases from negligible levels by up to 1000-fold after heat treatment (Lindquist 1980). Interestingly, unlike for the Transect populations, Hsp90 protein level decreased after heat shock in the Bonville lines. This difference was unexpected - possibly subtle uncontrolled differences in growth conditions may have led to this result, since the sets of flies were characterised at different times. Alternatively, the single Bonville population sample may have been a sampling oddity – differences may occur among samples from different natural populations, as occurred for example among populations from the Transect set with some showing low or no increase in Hsp90 protein following heat treatment (Fig. 4.4c). While our Hsp90 protein data showed significant effects of heat treatment no significant variation among lines or among populations was revealed, despite the latitudinal association detected. More replicates may have detected a line effect or perhaps our multi-gel Western blot method of quantification lacked sensitivity. Future efforts using ELISA methodology may improve resolution. The fact that we detected little association, within sets of lines or populations, between transcript or protein measured pre- and post- heat treatment, or between transcript and protein, may have been a consequence of significant genetic variation in *hsp90* expression-control elements within and between populations.

Despite these shortcomings strong and significant negative associations were detected between basal *hsp90* transcript and basal heat knockdown in two data sets (the Bonville lines and Transect populations). In the Coffs Harbour lines it was the heat-stimulated *hsp90* transcript level that was negatively associated with basal heat tolerance. In the fourth data set, the Hybrid lines, while all four transcript/tolerance regressions were negative none were significant,

although three were at borderline significance (Table 4.2). The Hsp90 protein/tolerance result did not show the significant associations as observed for the transcript data. However, the two comparisons that bordered on significance, of eight comparisons, were negative in direction (Table 4.2). Therefore an overall negative pattern of association has emerged across multiple data sets, including across different temperature treatments and in different laboratories. Note that in most data sets it is the basal level of transcript or protein that has suggested the negative associations with basal adult heat tolerance. Our data suggest that flies with a high basal level of *hsp90* expression are less heat tolerant.

A possible explanation for an association between *hsp90* expression and adult tolerance might be an upstream factor responsible for elevated *hsp90* levels independently leading to changes in adult tolerance, removing any direct influence of *hsp90* on tolerance. Alternatively, downstream effects could be responsible. Under this scenario a compelling explanation for these data is a direct role of Hsp90 since it is both an established universal negative modulator of the heat shock response, via its interaction with Hsf (Ali *et al.* 1998; Duina *et al.* 1998; Zou *et al.* 1998; Bharadwaj *et al.* 1999; Voellmy 2004) and a negative regulator of heat shock gene expression (Sawarkar *et al.* 2012). Hsf is the initial primary switch for the heat shock response (Wu 1995; Morimoto 1998; Pirkkala *et al.* 2001). Under normal conditions much of the cellular Hsf is held in a complex with Hsp90 and other chaperones decreasing its availability to activate transcription (Nadeau *et al.* 1993). Upon heat shock the unbound monomeric Hsf aggregates into trimers that bind to and up-regulate the heat shock response genes (Birch-Machin *et al.* 2005). Under this hypothesis low basal levels of Hsp90 protein result in faster gene transcription and lead to high heat tolerance. It would be basal tissue levels of Hsp90, immediately prior to heat shock that would be an important influence on the speed of the response. This idea is deserving of further investigation, for example using controlled genetic manipulative experiments to separately alter both Hsp90 and Hsf protein levels and test thermotolerance.

Given that *hsp90* level was negatively associated with heat tolerance in our early data and that heat tolerance clines with latitude (Sgrò *et al.* 2010) we measured *hsp90* transcript and protein expression in eight populations collected along a geographical gradient along the Australian east coast. Latitudinal studies can provide valuable insight into whether or not natural selection is acting on genes and traits (Endler 1977). The clinal patterns found here among these recently established laboratory populations from widespread field sites suggest that there are genetically based differences in levels of *hsp90* transcript and protein among field populations. Consistent with the idea that it is basal *hsp90* levels that have a negative effect on heat tolerance and underlie latitudinal changes in tolerance, it was the basal *hsp90* transcript and basal protein levels that were highest in high-latitude, low heat tolerance, temperate populations.

Given the multitude of Hsp90 client proteins that are involved in cell signaling and trait development (Rutherford and Lindquist 1998; Chiosis *et al.* 2013) Hsp90 has the potential to influence many other traits. Thus expression of *hsp90* could influence development, growth and body size (Pratt 1998), susceptibility to infection (Cowen and Lindquist 2005) and traits related to metabolism and ATP production (Falcone *et al.* 2005; Pratt *et al.* 2006), not to mention morphological traits (Rutherford and Lindquist 1998; Queitsch *et al.* 2002). The latitudinal gradient in *hsp90* expression could therefore be a determining factor that underlies other traits that cline with latitude as a result of climatic adaptation in this species. Given the conserved nature of *hsp90*, variation in its expression across diverse climatic regions in other wide-ranging species might be a fruitful area of investigation.

While the statistical significance level of many of the associations reported here are low, the consistency of their negative direction across four data sets, combined with the established

negative regulatory role of *hsp90* for the heat shock response, suggest that *hsp90* should be further investigated in the context of heritable variation in expression affecting heat tolerance in this and other species. Certainly more research is called for to establish whether there is a direct causal relationship between *hsp90* and heat tolerance. Finally, the latitudinal clinal patterns of expression of *hsp90* and its potential effect on numerous traits invite future research efforts to assess the role of this chaperone in the differential adaptation of *Drosophila* and other species to diverse climatic regions.

4.6 Acknowledgements

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CHAPTER 5

Overview and future directions

5.1 General

In order to adapt to changing environments, populations of organisms need to harbour heritable variation in life history, physiological and biochemical traits that enhance survival and reproductive performance in the stressful new environment (Williams *et al.* 2008; Hoffmann 2010; Hoffmann and Sgrò 2011). Current awareness of global warming has highlighted the need for vulnerable species to vary in heat tolerance, in order to adapt and persist and it is the underlying physiology and biochemistry of heritable heat tolerance variation in the ectothermic *Drosophila melanogaster* that has been the focus of this thesis.

Drosophila is now an established model for research into the genetic and physiological basis of climatic adaptation, particularly since latitudinal clines in heat tolerance have been well studied in this organism and can provide insights into the genes driving stress responses – the association of high heat tolerance with hot climates providing the *prima facie* case for an adaptive trait. While numerous thermotolerance candidate genes have been identified (Nielsen *et al.* 2006; Levine and Begun 2008; Turner *et al.* 2008; Kolaczkowski *et al.* 2011), and changes in gene expression in response to heat stress have been characterised across the genome (Sørensen *et al.* 2005; Levine *et al.* 2011), little has been revealed about the underlying changes involved in relevant physiology and cellular processes – about the mechanisms that drive adaptation to heat stress. The object of this study was to examine heat tolerance in populations known to cline for this adaptive trait and try to forge a link between thermal tolerance, physiological processes and underlying genes.

I chose to look at adult heat knockdown time (one of many possible measures of heat tolerance variation), since adaptive variation in this trait in *Drosophila* has been well characterised (Hoffmann *et al.* 2002; Sgrò *et al.* 2010). Although innumerable studies have investigated the heat shock response in a great number of organisms, fewer studies have united molecular and quantitative approaches. In this thesis I have used the experimentally tractable organism

Drosophila melanogaster to examine clinal variation in heat tolerance phenotypes. I focused on two candidate physiological/cellular processes that might underpin observed latitudinal clinal patterns, and on expression changes in two relevant candidate genes.

5.2 Protein synthesis as a physiological regulator of heat tolerance

In diverse organisms, changes occur in the rate and nature of protein production following heat exposure (reviewed in Sonna *et al.* 2002; Zhao and Jones 2012; Hasanuzzaman *et al.* 2013). Two predominant features of these changes, but by no means the only important changes (Sørensen *et al.* 2005), are the shutdown of general housekeeping proteins and the tight up-regulation of the Hsps (Lindquist 1980; Lakhotia *et al.* 1999; Johnson *et al.* 2009a). The extent to which this occurs in *Drosophila* has earlier been indicated to be important for heat tolerance (Alahiotis and Stephanou 1982; Stephanou *et al.* 1983). More recently, grouping of many candidate thermotolerance genes into the 'GO' categories *translation* and *regulation of transcription* highlights the importance of protein synthesis processes for heat-stressed organisms (Nielsen *et al.* 2006; Laayouni *et al.* 2007).

There is recent evidence for a relationship between rate of protein synthesis after heat shock and the increase in heat tolerance after mild heat shock (Johnson *et al.* 2009b). This finding provokes interest for not only investigation of the role of single Hsps as candidates for underlying heat tolerance mechanisms but also for the role of the entire cell protein-synthesis complement in influencing heat tolerance. The Johnson *et al.* (2009b) study utilised a set of isofemale lines from a single population to look for correlations between various measures of protein synthesis and heat tolerance. I wanted to explore this further to examine variation across natural populations taken from diverse thermal environments.

My hypothesis, investigated in Chapter two, was that protein synthesis rate is a factor influencing the latitudinal variation in heat tolerance and that protein synthesis would therefore

vary with latitude. My protein synthesis data supported this hypothesis – protein synthesis rates were higher in low latitude tropical populations (Cockerell *et al.* 2014). While this result is consistent with the simple idea that faster synthesis of heat-shock proteins leads to higher knockdown tolerance (and would help explain the latitudinal heat-tolerance cline) it was inconsistent with earlier studies suggesting a negative relationship between these variables (Johnson *et al.* 2009b). Therefore in Chapter three I further investigated the role of protein synthesis rate in heat tolerance using a set of lines derived by crossing populations from the extreme ends of the geographic range in eastern Australia. Such a hybrid population should incorporate the full spectrum of relevant genetic variation and might better reveal any relationship relevant to regional climatic differentiation in tolerance. The results further supported the idea that protein synthesis rate is important for tolerance to heat, since heat-stimulated rates of protein synthesis were again negatively associated with both adult basal and heat-hardened knockdown time (Cockerell *et al.* 2013). Basal protein synthesis rate was also negatively associated with hardened knockdown time. However in combination with my results from Chapter two the data are inconsistent with the idea that higher rates of protein synthesis in tropical populations contribute to their higher levels of heat tolerance. These results suggest that factors other than protein synthesis are of over-riding influence in contributing to the latitudinal clines in heat tolerance.

Still pursuing a connection between protein synthesis and heat tolerance I then focussed on *hsr-omega*. *Hsr-omega* variation has been associated with both adult heat and cold tolerance, with latitude and with protein synthesis rate (McColl *et al.* 1996; McKechnie *et al.* 1998; Lakhotia *et al.* 2001; Anderson *et al.* 2003; Collinge *et al.* 2008; Johnson *et al.* 2011). I looked for a clinal pattern in levels of the *hsr-omega* nuclear transcript, *omega-n*. I found a positive linear cline in basal *omega-n* transcript which, because of the reported effect of higher levels of *omega-n* reducing levels of synthesis of 'housekeeping' proteins (Johnson *et al.* 2011), is consistent with the lower levels of protein synthesis in higher-latitude colder populations. However this interpretation is over-simplistic because *omega-n* levels have been variously associated with both

heat and cold tolerance (McKechnie *et al.* 1998), and with variations in genotype including an inversion that clines latitudinally (Anderson *et al.* 2005), and the associations are not always in a direction consistent with the above idea, as discussed in Chapter three. However, detection of the latitudinal clines in levels of *omega-n* suggests that the transcript is indeed important for climatic adaptation, perhaps influencing post-heat-stress reproductive traits such as male fertility and female egg production, particularly given the high levels of *omega-n* reported in *Drosophila* testes and ovaries (Mutsuddi and Lakhotia 1995).

5.3 *Hsp90* gene contribution to adaptive heat tolerance variation

Hsp90 is a ubiquitously expressed chaperone molecule, is modestly up-regulated upon heat stress and has not yet been seriously investigated for its role in heat tolerance variation. In Chapter four I looked for differences in *hsp90* expression among lines and populations that have genetically diverged for heat tolerance. I found that basal *hsp90* levels significantly differed between populations from different geographical regions, suggesting a link to climatic adaptation. I also examined the link between *hsp90* and heat tolerance and found consistent significant negative associations between *hsp90* transcript or protein level and adult heat tolerance, in three independent sets of family lines. These data are consistent with recent *in vitro* work that has illuminated how Hsp90 protein, the most abundant Hsp in normal cells, exerts a negative regulatory role in the heat shock response (Ali *et al.* 1998; Duina *et al.* 1998; Zou *et al.* 1998; Bharadwaj *et al.* 1999; Duncan 2005). Under normal conditions Hsp90 is in a complex with the principal heat shock gene transcription factor, Hsf. This effectively inactivates Hsf, thus minimising high heat-shock gene transcription. Under this model low levels of Hsp90 would inactivate less Hsf leaving higher cellular levels of Hsf available to more quickly initiate the heat shock response, and perhaps lead to higher knockdown tolerance. Also, a direct negative regulatory role for Hsp90 in transcription of many stress response genes (Sawarkar *et al.* 2012), predicts that higher levels of Hsp90 protein would inhibit such transcription and thus be negatively associated with heat tolerance.

I found positive latitudinal clines in basal *hsp90* transcript and protein levels among populations collected from the Australian east coast - tropical populations expressed less *hsp90* transcript and protein than temperate populations. Thus I have shown that this important gene, with roles in many cellular processes (Csermely *et al.* 1998; Zuehlke and Johnson 2009), may also underlie at least part of the natural genetic variation in heat tolerance both within and among populations. At the very least variation in levels of *hsp90* transcript and protein across climatic gradients suggests that this gene is an important component of the mechanisms that facilitate differential adaptation to climatic regions.

Although I found several independent associations between *hsp90* and heat tolerance more evidence will be required to demonstrate a causal role of Hsp90 protein in this relationship to heat tolerance. Since Hsp90 is abundant in all cells and a total knockout *hsp90* is therefore likely to be inviable (Solit and Chiosis 2008), one step towards this end could be by reducing *hsp90* expression in a specific tissue, or group of tissues such as muscle or neuronal tissue, using a technique such as RNAi (Fire *et al.* 1998), and testing for heat knockdown tolerance. Also, investigating the role of natural variation in levels of Hsf as a determinant of thermal tolerance variation would be an important research direction. Is heat tolerance directly proportional to the amount of active/inactive Hsf protein? Available data using a temperature dependant *hsf* mutant line has provided ambiguous evidence for an effect of *hsf* on adult heat tolerance (Nielsen *et al.* 2005; Sørensen *et al.* 2005). We know that Hsf is activated differently among *Drosophila* populations reared at different temperatures (18 °C, 25 °C and 28 °C; Lerman and Feder 2001). Strains reared at high temperatures are slower to activate Hsf than those reared at lower temperatures. Therefore an effort needs to be made to bring together new molecular approaches with adaptive evolutionary biology to show the full scope of how *hsp90* and *hsf* genes or proteins affect heat tolerance variation in natural populations.

5.4 Protein synthesis and Hsp90

Hsp90 associates with many different proteins in normal and heat-affected cells (Chiosis *et al.* 2013), but particularly relevant to this study is its interaction with the heme-regulated inhibitor (HRI), an eIF-2α kinase, which plays a role in regulating protein synthesis when cells are stressed (Rose *et al.* 1989). By interacting with HRI, higher levels of Hsp90 protein effectively inhibit protein synthesis (Pal 1998). This interaction at the cellular level suggests that the two processes implicated to influence heat-knockdown tolerance may not be independent. Future study to jointly assess protein synthesis, HRI and *hsp90* levels for any effects on thermal tolerance across a set of single family lines will be important. If such an effect were present the negative latitudinal cline in rate of total protein synthesis presented in Chapter two and the positive latitudinal cline in basal level of *hsp90* transcript and protein (Chapter four) could also be causally related, since high levels of Hsp90 protein increase activity of the heme-controlled eIF-2a kinase and therefore decrease rate of protein synthesis (Rose *et al.* 1989). Thus it would be interesting to look further into this in the context of natural populations and measure protein synthesis rate, *hsp90* level and HRI levels across a set of populations from a climatically diverse transect.

5.5 A trade-off between heat and cold tolerance

This thesis has focused primarily on two specific physiological and genetic mechanisms underlying heat tolerance. However adaptation to cool temperate climatic regions has occurred in this species over the last one hundred or so years, since *D. melanogaster* colonised much of the world from tropical Africa (Hoffmann and Weeks 2007). For example, in Northern America cool latitude populations are now particularly capable of adult reproductive diapause during the winter months (Schmidt and Paaby 2008). Extensive genetic differentiation across the genome has facilitated this spread (Turner *et al.* 2008; Fabian *et al.* 2012; Telonis-Scott *et al.* 2013). Since *hsps*, including *hsp90*, are also up-regulated under cold stress conditions (Colinet *et al.*

2010), since cold tolerance also clines latitudinally along the Australian eastern coast (Hoffmann *et al.* 2002), and since *hsr-omega* variation has been associated with cold treatments (Collinge *et al.* 2008), one direction to take this research is to further explore how protein synthesis rate, *hsr-omega* and *hsp90* relate to cold tolerance and cold exposure. Possibly, in adapting to cold, the expression of such genes has been compromised for optimal performance under heat stress, and provides a reason for the lower heat tolerance of flies from cool temperate regions. This trade-off idea has been previously suggested. Specifically, a trade-off between heat and cold tolerance has been associated with a genetic element on chromosome 3, perhaps *In(3R)P* that harbours the *hsr-omega* gene (Anderson *et al.* 2003; Anderson *et al.* 2005). Thus even though some high latitude populations experience severe heat stress in summer (Parkash and Munjal 1999), when one might expect high tolerance to be valuable, the trade-off with higher cold tolerance enabling persistence through winter may have precluded the evolution of high heat tolerance in these habitats. Measuring cold-stressed flies for protein synthesis rates, *hsp90* transcript and protein level, and *omega-n* transcript level in populations from climatically diverse regions may help to further elucidate the role of these factors and changes to thermal tolerance mechanisms across climatic regions.

5.6 Summary and conclusions

This PhD thesis expands on our understanding of the underlying physiology and biochemistry of heat tolerance that varies adaptively across climatically diverse populations of the model organism *Drosophila*. The research provides insight into the biochemical control of the cellular heat shock response, perhaps the most conserved and important mechanism that facilitates heat stress resistance. Two aspects of this control are the focus of the study; i) the role of protein synthesis changes that occur upon heat stress, and ii) the role of a ubiquitous and constitutive heat shock protein, Hsp90.

Taken together the results of this thesis highlight the likely contributions of protein synthesis rate, *omega-n* and *hsp90* to adaptive heat tolerance variation in natural populations of *Drosophila*. These cellular processes and genes need to be investigated further in the context of climatic adaptation, in this and other ectothermic species, using an inter-disciplinary approach that involves molecular biology, biochemistry, physiology and evolutionary biology.

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