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

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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.



## 1. 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., Bahrndorff et al., 2006; Kuo and Sanford, 2009; Zani et al., 2005). 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

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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; Sorensen 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 (Sorensen et al., 2005; Kültz, 2005). However, in *Drosophila* within the first 2 h 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., 2009a). We know that Hsps are quickly up-regulated following a mild heat stress, that they have diverse modes of action (Feder and Hoffman, 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., 2009b). *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 our 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 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 characterise clinal populations for protein synthesis levels both before and following a mild heat stimulus, and after rearing at both 18 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. Materials and methods

### 2.1. 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.

### 2.2. 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 and 25 °C) 30 females were tested randomly over 10 runs over 2 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- 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 we could explicitly examine the effect of rearing temperature on heat tolerance.

### 2.3. 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. (2009b, 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 3 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., 2011), 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).

### 2.4. 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. (2011). 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 normalised 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).

### 2.5. 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.

## 3. Results

### 3.1. Heat knockdown time

Rearing temperature and hardening treatment had significant effects on heat tolerance (Table 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. 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 and 25 °C – tropical populations were more heat tolerant after heat hardening than temperate populations (Fig. 1, Table 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. 1b, Table 2).

### 3.2. Protein synthesis rates

Significant variation amongst 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 and 25 °C (Table 1, Fig. 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 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. 2a, Table 2). However, following 18 °C rearing heat-stimulated protein synthesis did not show any clinal variation (Fig. 2b, Table 2). In contrast, when rearing occurred at 25 °C a cline was not detected for basal protein synthesis (Fig. 2c, Table 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. 2d).

Amongst the populations from across the geographic range correlation between protein synthesis rate and heat tolerance revealed only two positive associations (Table 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.

### 3.3. 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 1, Fig. 3). There was also a significant effect of population, and a population by heat stimulus interaction (Table 1, Fig. 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). 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 = $-4.589 \pm 1.478$, $b = 0.077$, $p = 0.027$; Fig. 3). Populations from either end of the cline had higher levels of *omega-n* than mid-latitude populations.

## 4. Discussion

### 4.1. Heat tolerance

Our data confirm that rearing at cooler temperatures results in a decrease in heat tolerance in accordance with previous reports

**Table 1**
Analyses of variance testing heat knockdown time, protein synthesis (reared at 18 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 (min) | Rearing temperature | 1 | 33,560.17 | 1326.89 | <0.0001 |
| | Hardening treatment | 1 | 22,417.11 | 886.32 | <0.0001 |
| | Population | 17 | 2881.66 | 6.70 | <0.0001 |
| | Run | 9 | 2098.03 | 9.22 | <0.0001 |
| | Rearing temperature × hardening treatment | 1 | 691.41 | 27.34 | <0.0001 |
| | Rearing temperature × population | 17 | 1442.68 | 3.36 | <0.0001 |
| | Hardening treatment × population | 17 | 754.24 | 1.75 | 0.0285 |
| | Rearing temperature × hardening treatment x Population | 17 | 832.86 | 1.94 | 0.0120 |
| | Error | 1904 | 48,156.79 | | |
| Protein synthesis rate (18 °C) | Heat treatment | 1 | 0.20 | 0.00 | 0.9829 |
| | Population | 17 | 13,105.21 | 1.84 | 0.0421 |
| | Heat treatment × population | 15 | 6060.87 | 0.96 | 0.5017 |
| | Error | 63 | 26,394.29 | | |
| Protein synthesis rate (25 °C) | Heat treatment | 1 | 1222.01 | 2.23 | 0.1418 |
| | Population | 13 | 17,047.62 | 2.39 | 0.0134 |
| | Heat treatment × population | 14 | 4220.65 | 0.64 | 0.7979 |
| | Error | 52 | 28,557.02 | | |
| Omega-n transcript level | Population | 15 | 1382.86 | 5.30 | <0.0001 |
| | Heat treatment | 1 | 1646.19 | 94.62 | <0.0001 |
| | Population × heat treatment | 15 | 1283.80 | 4.92 | <0.0001 |
| | Error | 60 | 1043.85 | | |

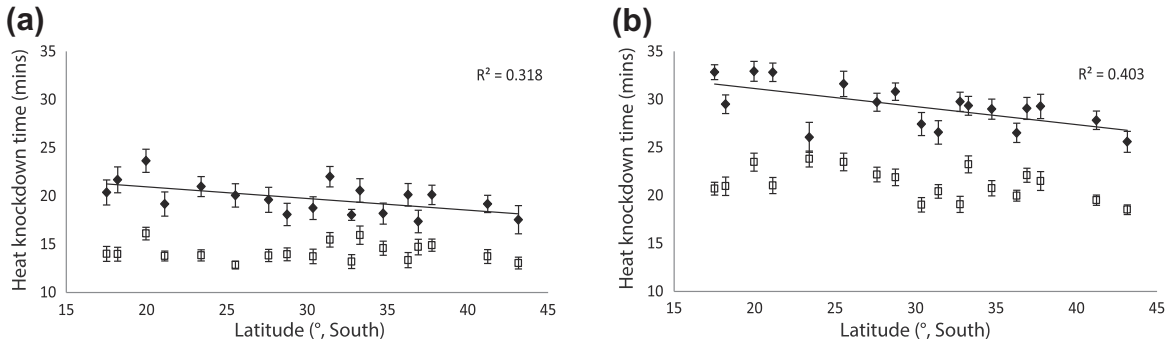


**Fig. 1.** Latitudinal patterns of heat tolerance in *D. melanogaster* from the Australian east coast (2008 collection). Open squares represent basal knockdown time and filled diamonds represent hardened heat knockdown time. Error bars ± standard error. (a) Basal and hardened heat knockdown time when reared at 18 °C, (b) basal and hardened heat knockdown time when reared at 25 °C.

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

(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 7 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 and 25 °C.

### 4.2. 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., 1982; 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 our 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. One might have expected a cline in the opposite direction given the normal physiological compensatory effects associated with adaptation to colder regions

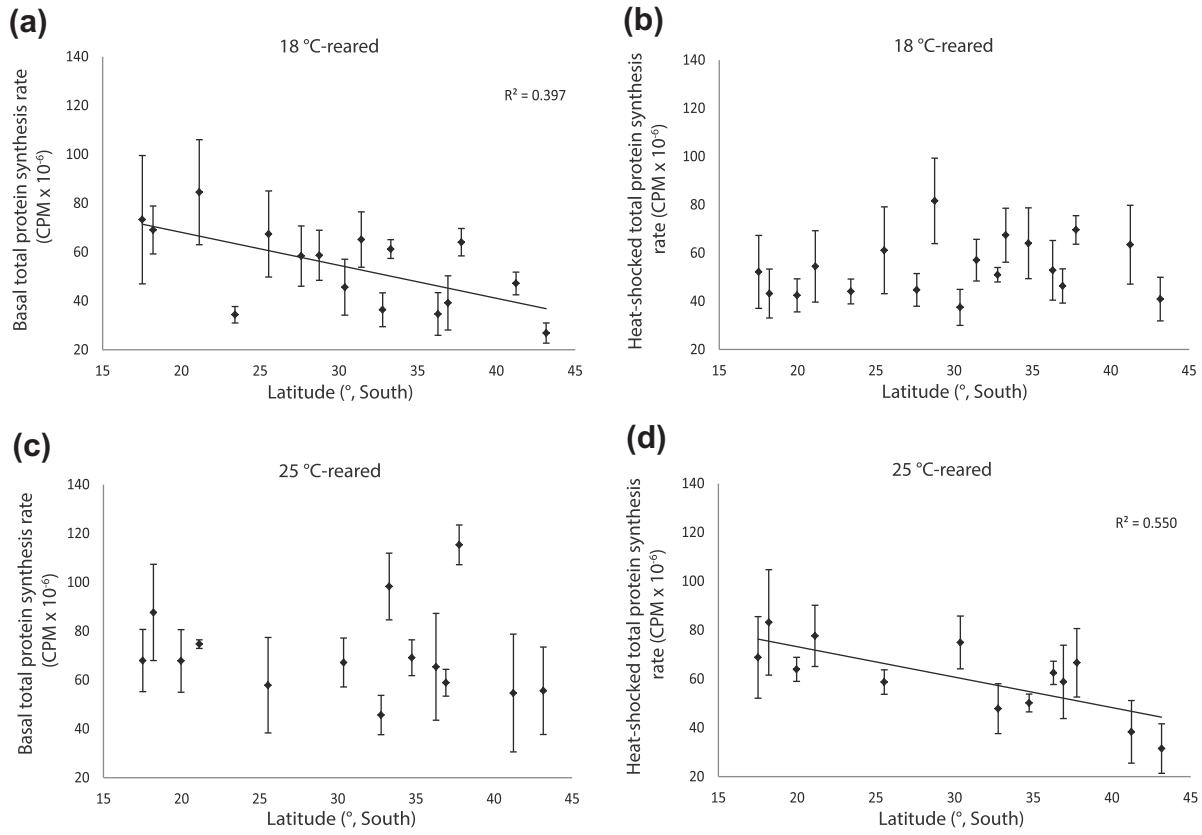


**Fig. 2.** Latitudinal patterns of total protein synthesis rates in *D. melanogaster* females (2008 collection), (a) reared at 18 °C with no heat treatment, (b) reared at 18 °C with heat stimulus, (c) reared at 25 °C with no heat treatment, and (d) reared at 25 °C with heat stimulus. Error bars ± standard error.

**Table 3**
Associations between rates of protein synthesis and heat knockdown across the 2008 collection of populations of *D. melanogaster* reared at 18 and 25 °C.

| Rearing temperature (°C) | Protein synthesis measure | Heat tolerance measure | *r* | *p*-Value |
|---|---|---|---|---|
| 18 | Basal | Basal | 0.304 | 0.126 |
| | | Hardened | 0.457 | 0.038* |
| 25 | Basal | Basal | 0.168 | 0.253 |
| | | Hardened | −0.152 | 0.274 |
| 18 | Heat-stimulated | Basal | 0.383 | 0.088 |
| | | Hardened | 0.109 | 0.356 |
| 25 | 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.

(Frazier et al., 2006; Clarke, 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 3), so this idea should not be discarded.

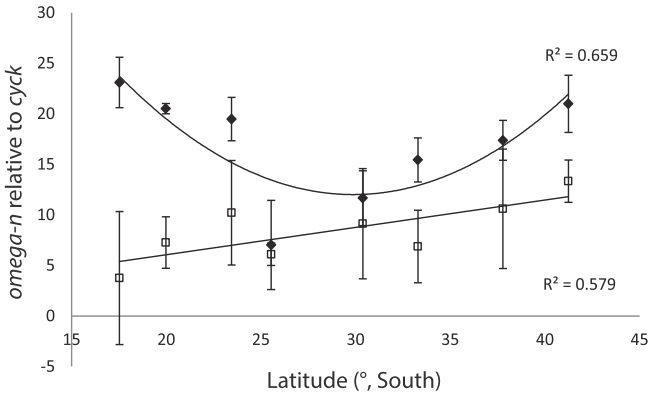


**Fig. 3.** Latitudinal patterns of *omega-n* transcript level in 25 °C-reared populations (2009 collection) measured following heat stimulus (filled diamonds), or measured without heat stimulus (open squares). Error bars ± standard error.

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., 2009a).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. 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, *Caenorhabditis 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., 2009b), 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).

### 4.3. 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; Sarup et al., 2006; Sgrò and Blows, 2003; 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), 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. (2009b), 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., 2009b), 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).

## 5. 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.

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## References

Anderson, A.R., Collinge, J.E., Hoffmann, A.A., Kellett, M., McKechnie, S.W., 2003. Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*. Heredity 90, 195–202.

Anderson, A.R., Hoffmann, A.A., McKechnie, S.W., Umina, P.A., Weeks, A.R., 2005. The latitudinal cline in the *In(3R)Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations. *Molecular Ecology* 14, 851–858.

Azevedo, R.B.R., French, V., Partridge, L., 1996. Thermal evolution of egg size in *Drosophila melanogaster*. Evolution 50, 2338–2345.

Bahrndorff, S., Holmstrup, M., Petersen, H., Loeschcke, V., 2006. Geographic variation for climatic stress resistance traits in the springtail *Orchesella cincta*. *Journal of Insect Physiology* 52, 951–959.

Berrigan, D., Partridge, L., 1997. Influence of temperature and activity on the metabolic rate of adult *Drosophila melanogaster*. *Comparative Biochemistry and Physiology* 118A, 1301–1307.

Bownes, M., 1980. Ovarian synthesis of yolk proteins in *Drosophila melanogaster*. Genetika 12, 13–20.

Brattstrom, B.H., 1970. Thermal acclimation in Australian amphibians. *Comparative Biochemistry and Physiology* 35, 69–103.

Cavicchi, S., Guerra, D., La Torre, V., Huey, R.B., 1995. Chromosomal analysis of heat-shock tolerance in *Drosophila melanogaster* evolving at different temperatures in the laboratory. Evolution 49, 676–684.

Chown, S.L., Terblanche, J.S., 2007. Physiological diversity in insects: ecological and evolutionary contexts. In: Simpson, S.J. (Ed.), Advances in Insect Physiology. Academic Press Ltd, Elsevier Science Ltd, London.

Clarke, A., 2006. Temperature and the metabolic theory of ecology. *Functional Ecology* 20, 405–412.

Cockerell, F.E., Sgro, C.M., McKechnie, S.W., 2013. Capacity for protein synthesis following heat stimulus of *Drosophila* associates with heat tolerance but does not underlie the latitudinal tolerance cline. *Journal of Thermal Biology* 38, 524–529.

Collinge, J.E., Anderson, A.R., Weeks, A.R., Johnson, T.K., McKechnie, S.W., 2008. Latitudinal and cold-tolerance variation associate with DNA repeat-number variation in the *hsr-omega* RNA gene of *Drosophila melanogaster*. Heredity 101, 260–270.

Cooper, B.S., Tharp, J.M., Jernberg, I.I., Angilletta Jr., M.J., 2012. Developmental plasticity of thermal tolerances in temperate and subtropical populations of *Drosophila melanogaster*. *Journal of Thermal Biology* 37, 211–216.

David, J.R., Gibert, P., Moreteau, B., Gilchrist, G.W., Huey, R.B., 2003. The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in *Drosophila subobscura*. *Functional Ecology* 17, 425–430.

Deutsch, C.A., Tewskbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C., Martin, P.R., 2008. Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Science* USA 105, 6668–6672.

DiDomenico, B.J., Bugaisky, G.E., Lindquist, S., 1982. The heat shock response is self regulated at both the transcriptional and post-transcriptional levels. Cell 31, 593–603.

Endler, J.A., 1977. Geographic Variation, Speciation, and Clines. Princeton University Press, Princeton, NJ.

Fallis, L.C., Fanara, J.J., Morgan, T.J., 2011. Genetic variation in heat-stress tolerance among South American *Drosophila* populations. Genetica 139, 1331–1337.

Feder, M.E., Hoffman, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61, 243–282.

Frazier, M.R., Huey, R.B., Berrigan, D., 2006. Thermodynamics constrains the evolution of insect population growth rates: "warmer is better". *The American Naturalist* 168, 512–520.

Ghalambor, C.K., Huey, R.B., Martin, P.R., Tewksbury, J.J., Wang, G., 2006. Are mountain passes higher in the tropics? Janzen's hypothesis revisited. *Integrative and Comparative Biology* 46, 5–17.

Goldberg, A.L., 2003. Protein degradation and protection against misfolded or damaged proteins. Nature 426, 895–899.

Guerra, D., Cavicchi, S., Krebs, R.A., Loeschcke, V., 1997. Resistance to heat and cold stress in *Drosophila melanogaster*: intra and inter population variation in relation to climate. Genetics Selection Evolution 29, 497–510.

Hallas, R., Schiffer, M., Hoffmann, A.A., 2002. Clinal variation in *Drosophila serrata* for stress resistance and body size. Genetics Research 79, 141–148.

Hoffmann, A.A., 2010. Physiological climatic limits in *Drosophila:* patterns and implications. The Journal of Experimental Biology 213, 870–880.

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

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

Hoffmann, A.A., Sorensen, J.G., Loeschcke, V., 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* 28, 175–216.

Hoffmann, A.A., Shirriffs, J., Scott, M., 2005. Relative importance of plastic *vs* genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Functional Ecology* 19, 222–227.

Hogan, N.C., Slot, F., Traverse, K.L., Garbe, J.C., Bendena, W.G., Pardue, M.L., 1995. Stability of tandem repeats in *Drosophila melanogaster Hsr-omega* nuclear RNA. Genetics 139, 1611–1621.

James, A.C., Partridge, L., 1995. Thermal evolution of the rate of larval development in *Drosophila melanogaster* in lab and field populations. Journal of Evoltionary Biology 8, 315–330.

Johnson, T.K., Cockerell, F.E., Carrington, L.B., Rako, L., Hoffmann, A.A., McKechnie, S.W., 2009a. The capacity of *Drosophila* to heat harden associates with low rates of heat-shocked protein synthesis. *Journal of Thermal Biology* 34, 327–331.

Johnson, T.K., Carrington, L.B., Hallas, R., McKechnie, S.W., 2009b. Protein synthesis rates in *Drosophila* associate with levels of the *hsr-omega* nuclear transcript. Cell Stress and Chaperones 14, 569–577.

Johnson, T.K., Cockerell, F.E., McKechnie, S.W., 2011. Transcripts from the *Drosophila* heat-shock gene *hsr-omega* influence rates of protein synthesis but hardly affect resistance to heat knockdown. *Molecular Genetics and* Genomics 285, 313–323.

Kellett, M., Hoffmann, A.A., McKechnie, S.W., 2005. Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. *Functional Ecology* 19, 853–858.

Klopfer, P.H., MacArthur, R.H., 1960. Niche size and faunal diversity. *The American Naturalist* 94, 293–300.

Kolaczkowski, B., Kern, A.D., Holloway, A.K., Begun, D.J., 2011. Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. Genetics 187, 245–260.

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology* 67, 225–257.

Kuo, E.S., Sanford, E., 2009. Geographic variation in the upper thermal limits of an intertidal snail: implications for climate envelope models. *Marine Ecology Progress Series* 388, 137–146.

Laayouni, H., Garcia-Franco, F., Chavez-Sandoval, B.E., Trotta, V., Beltran, S., Corominas, M., Santos, M., 2007. Thermal evolution of gene expression profiles in *Drosophila subobscura*. BioMedical Central Evolutionary Biology 7, 42–56.

Lakhotia, S.C., 2011. Forty years of the 93D puff of *Drosophila melanogaster*. Journal of Bioscience 36, 399–423.

Lee, S.F., Chen, Y., Varan, A.K., Wee, C.W., Rako, L., Axford, J.K., Good, R.T., Blacket, M.J., Reuter, C., Partridge, L., Hoffmann, A.A., 2011. Molecular basis of adaptive shift in body size in *Drosophila melanogaster*: functional and sequence analyses of the *Dca* gene. *Molecular Biology and Evolution* 28, 2393–2402.

Leemans, R., Eggere, B., Loop, T., Kammermeier, L., Haiqiong, H., Hartmann, B., Certa, U., Hirth, F., Reichert, H., 2000. Quantitative transcript imaging in normal and heat-shocked *Drosphila* embryos by using high density oligonucleotide arrays. *Proceedings of the National Academy of Science* 97, 12138–12143.

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

Magiafoglou, A., Carew, M.E., Hoffmann, A.A., 2002. Shifting clinal patterns and microsatellite variation in *Drosophila serrata* populations: a comparison of populations near the southern border of the species range. *Journal of Evolutionary Biology* 15, 763–774.

Marygold, S.J., Roote, J., Rueter, G., Lambertsson, A., Ashburner, M., Millburn, G.H., Harrison, P.M., Yu, Z., Kenmochi, N., Kaufman, T.C., Leevers, S.J., Cook, K.R., 2007. The ribosomal protein genes and *minute* loci of *Drosophila melanogaster*. Genome Biology 8, R216.

McColl, G., Hoffmann, A.A., McKechnie, S.W., 1996. Response of two heat shock genes to selection for knockdown heat resistance in *Drosophila melanogaster*. Genetics 145, 1615–1627.

McKechnie, S.W., Halford, M.M., McColl, G., Hoffmann, A.A., 1998. Both allelic variation and expression of nuclear and cytoplasmic transcripts of *hsr-omega* are closely associated with thermal phenotype in *Drosophila*. Proceedings of the National Academy of Science 95, 2423–2428.

Mitrovski, P., Hoffmann, A.A., 2001. Postponed reproduction as an adaptation to winter conditions in Drosophila melanogaster: evidence for clinal variation under semi-natural conditions. Proceedings Biological Sciences 268, 2163–2168.

Mutsuddi, M., Lakhotia, S.C., 1995. Spatial expression of the *hsr-omega (93D)* gene in different tissues of *Drosophila melanogaster* and identification of promoter controlling its developmental expression. *Developmental Genetics* 17, 308–311.

Overgaard, J., Kristensen, K.V., Mitchell, K.A., Hoffmann, A.A., 2011. Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *The American Naturalist* 178, S80–S96.

Pardue, M.L., Bendena, W.G., Fini, M.E., Garbe, J.C., Hogan, N.C., Traverse, K.L., 1990. *Hsr-omega,* a novel gene encoded by a *Drosophila* heat shock puff. Biology Bulletin 179, 77–86.

Parmesan, C., 1996. Climate and species range. Nature 382, 765–766.

Parsell, D., Lindquist, S., 1993. The function of heat shock proteins in stress tolerance: degradation and re-activation of damaged proteins. Annual Review of Genetics 27, 437–496.

Rako, L., Blacket, M.J., McKechnie, S.W., Hoffmann, A.A., 2007. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. *Molecular Ecology* 16, 2948–2957.

Rako, L., Poulsen, A., Shirriffs, J., Hoffmann, A.A., 2009. Clinal variation in post-winter male fertility retention; an adaptive overwintering strategy in *Drosophila melanogaster*. *Journal of Evolutionary Biology* 22, 2438–2444.

Sarup, P., Sorensen, J.G., Dimitrov, K., Barker, J.S.F., Loeschcke, V., 2006. Climatic adaptation of *Drosophila buzzatii* populations in southeast Australia. Heredity 96, 479–486.

Saunders, D.S., Gilbert, L.I., 1990. Regulation of ovarian diapause in *Drososphila melanogaster* by photoperiod and moderately low temperature. *Journal of Insect Physiology* 36, 195–200.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative $C_T$ method. *Nature Protocols* 3, 1101–1108.

Sgrò, C.M., Blows, M.W., 2003. Evolution of additive and nonadditive genetic variance in development time along a cline in *Drosophila serrata*. Evolution 57, 1846–1851.

Sgrò, C.M., Overgaard, J., Kristensen, K.V., Mitchell, K.A., Cockerell, F.E., Hoffmann, A.A., 2010. A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from eastern Australia. *Journal of Evolutionary Biology* 23, 2484–2493.

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

Stephanou, G., Alahiotis, S.N., Christodoulou, C., Marmaras, V.J., 1983. Adaptation of *Drosophila* to temperature: heat-shock proteins and survival in *Drosophila melanogaster*. Developmental Genetics 299–308.

Storti, R.V., Scott, M.P., Rich, A., Pardue, M.L., 1980. Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. Cell 22, 825–834.

Syntichaki, P., Troulinaki, K., Tavernarakis, N., 2007. Protein synthesis is a novel determinant of aging in *Caenorhabditis elegans*. Annual New York Academy of Sciences 1119, 289–295.

Tavernarakis, N., 2008. Ageing and the regulation of protein synthesis: a balancing act? Trends in Cell Biology 18, 228–235.

Teleman, A.A., Chen, Y.-W., Cohen, S.M., 2005. 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. Genes and Development 19, 1844–1848.

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

Terblanche, J.S., Hoffmann, A.A., Mitchell, H.K., Rako, L., le Roux, P.C., Chown, S.L., 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. The Journal of Experimental Biology 214, 3713–3725.

Trotta, V., Calboli, F.C., Ziosi, M., Guerra, D., Pezzoli, M.C., David, J.R., Cavicchi, S., 2006. Thermal plasticity in *Drosophila melanogaster:* a comparison of geographic populations. BioMedical Central Evolutionary Biology 6, 67.

Umina, P.A., Hoffmann, A.A., Weeks, A.R., McKechnie, S.W., 2006. An independent non-linear latitudinal cline for the sn-glycerol-3-phosphate (α-*Gpdh*) polymorphism of *Drosophila melanogaster* from eastern Australia. Genetics Research 87, 13–21.

van Heerwaarden, B., Lee, R.F.H., Wegener, B., Weeks, A.R., Sgrò, C.M., 2012. Complex patterns of local adaptation in heat tolerance in *Drosophila simulans* from eastern Australia. Journal of Evolutionary Biology 25, 1765–1778.

West-Eberhard, M.J., 1989. Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics 20, 249–278.

Yost, H.J., Petersen, R.B., Lindquist, S., 1990. Post-transcriptional regulation of heat shock protein synthesis in Drosophila. In Stress Proteins and Biology and Medicine, Cold Spring Harbour Laboratory Procs., pp. 379–409.

Zani, P.A., Swanson, S.E., Corbin, D., Cohnstaedt, L.W., Agotsch, M.D., Bradshaw, W.E., Holzapfel, C.M., 2005. Geographic variation in tolerance of transient thermal stress in the mosquito *Wyeomia smithii*. Ecology 86, 1206–1211.