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Complex patterns of local adaptation in heat tolerance in *Drosophila simulans* from eastern Australia

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Abstract

Latitudinal clines are considered a powerful means of investigating evolutionary responses to climatic selection in nature. However, most clinal studies of climatic adaptation in *Drosophila* have involved species that contain cosmopolitan inversion polymorphisms that show clinal patterns themselves, making it difficult to determine whether the traits or inversions are under selection. Further, although climatic selection is unlikely to act on only one life stage in metamorphic organisms, a few studies have examined clinal patterns across life stages. Finally, clinal patterns of heat tolerance may also depend on the assay used. To unravel these potentially confounding effects on clinal patterns of thermal tolerance, we examined adult and larval heat tolerance traits in populations of *Drosophila simulans* from eastern Australia using static and dynamic (ramping 0.06 °C min^{-1}) assays. We also used microsatellites markers to clarify whether demographic factors or selection are responsible for population differentiation along clines. Significant cubic clinal patterns were observed for adult static basal, hardened and dynamic heat knockdown time and static basal heat survival in larvae. In contrast, static, hardened larval heat survival increased linearly with latitude whereas no clinal association was found for larval ramping survival. Significant associations between adult and larval traits and climatic variables, and low population differentiation at microsatellite loci, suggest a role for climatic selection, rather than demographic processes, in generating these clinal patterns. Our results suggest that adaptation to thermal stress may be species and life-stage specific, complicating our efforts to understand the evolutionary responses to selection for increasing thermotolerance.

Introduction

It has long been recognized that the distribution and abundance of many species, particularly ectotherms, is influenced by temperature (Cossins & Bowler, 1987). As evidence for anthropogenic climate change increases, interest is focussing on adaptation to upper thermal limits. A relationship between upper thermal tolerance and maximum habitat temperature has been demonstrated for many species (Tomanek & Somero, 1999; Stillman & Somero, 2000; Stillman, 2002; Compton *et al.*, 2007; Sinervo *et al.*, 2010; Somero, 2010; Duarte *et al.*, 2012), suggesting that some ectotherms already exist close to upper thermal thresholds (Stillman, 2002; Mercader & Scriber, 2008; Jones *et al.*, 2009). Insight into selection for upper thermal limits can be gained by testing for clinal patterns in heat tolerance in ectotherms when populations are tested in common environments that can be linked back to habitat temperatures (Hoffmann *et al.*, 2003; Somero, 2010).

The wide range of distributions (from cosmopolitan to restricted) and thermal niches that *Drosophila* species occupy makes them an ideal model system to examine evolutionary responses to temperature extremes (Hoffmann *et al.*, 2003). A powerful means of investigating evolutionary responses within species is to examine clinal/geographical variation in traits between natural

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populations. Clinal variation along natural climatic gradients can be useful for identifying traits and genes associated with certain environmental conditions (Endler, 1977) and provide strong evidence for climatic selection when patterns are understood in terms of effects on fitness. Latitudinal clines in a number of quantitative traits including body size, development time, starvation resistance and, most importantly, heat and cold tolerance (reviewed in (Hoffmann & Weeks, 2007) have been observed in a number of *Drosophila* species, providing indirect evidence for thermal adaptation in nature. Such clinal patterns enable the investigation of the physiological and ecological factors contributing to the evolution of thermotolerance in nature.

Opposing linear clines in heat and cold resistance have been observed in *Drosophila melanogaster* populations along the east coast of Australia (Hoffmann *et al.*, 2002). Knockdown time following heat shock increased towards the tropics, whereas recovery time following cold shock decreased towards temperate latitudes. These clinal patterns in static heat knockdown time have since been confirmed (Sgrò *et al.*, 2010). Although heat knockdown time has also been associated with environmental temperature (altitudinal and latitudinal variation) in *D. buzzatii* (Parkash *et al.*, 2005; Sarup *et al.*, 2006, 2009), no such clinal patterns (latitudinal or altitudinal) have been found in *D. simulans* (Arthur *et al.*, 2008; Sarup *et al.*, 2009). *Drosophila simulans* is closely related to *D. melanogaster* and both species share a similarly cosmopolitan distribution. These species differences in clinal patterns in thermal tolerance may reflect different mechanisms of thermal adaptation, or a greater role for population demographic processes such as drift, gene flow or colonization history in *D. simulans*.

In addition, recent studies suggest that evidence for adaptive shifts and clinal patterns in thermotolerance can depend on the type of assay used (Kelty & Lee, 2001; Terblanche *et al.*, 2007). Thermal tolerance can be assessed using either static (constant temperature) (Hoffmann *et al.*, 2002, 2003) or dynamic (variable temperature) assays (Terblanche *et al.*, 2007). Recent debate has focussed on using so-called ecologically relevant measures (Kelty & Lee, 2001; Terblanche *et al.*, 2007, 2011; Rezende *et al.*, 2011; Santos *et al.*, 2011; Overgaard *et al.*, 2012) and one method that is increasingly being used involves gradually heating or cooling an animal from a particular starting temperature until physiological failure, such as knockdown or loss of righting ability (Terblanche *et al.*, 2007; Mitchell & Hoffmann, 2010; Sgrò *et al.*, 2010) These measures are argued to be more ecologically relevant because they are thought to better reflect changes in temperature in the field (Terblanche *et al.*, 2007). However, data suggest that the outcome of these assays can also be influenced by the rate of temperature changes (Kelty & Lee, 2001; Terblanche *et al.*, 2007; Sgrò *et al.*, 2010), which might confound or obscure any geographical or clinal patterns that are present.

Of particular relevance to this current study, Sgrò *et al.* (2010) found that latitudinal patterns in heat tolerance in populations of *D. melanogaster* from eastern Australia depended on the rate at which temperature was increased; a significant linear cline, which paralleled those found for static basal and hardened knockdown time, was found when temperature was increased at a rate of 0.06 °C min^{-1}, but not at 0.1 °C min^{-1}. It has recently been argued (Rezende *et al.*, 2011; Santos *et al.*, 2011) that ramping rates in experiments aiming to study the evolutionary basis of thermal tolerance should be as fast as possible to avoid decreases in fly physical condition (due to starvation and desiccation stress) during slower ramping assays that will confound conclusions about adaptive differences in thermal tolerance between populations. However, mean shaded heating rates of 0.06 °C min^{-1} have been observed on days exceeding 35 °C in south-eastern Victoria, Australia, whereas the faster rate of increase of 0.1 °C min^{-1} was not observed even when the maximum daily temperature exceeded 40 °C (Fig. S1, www.bom.gov.au), which occurs on average 0 to 1.1 days per year at the sites we sampled along the east coast of Australia. Thus, the rate of 0.06 °C min^{-1} seems more likely to be experienced at least by the adults and larvae of the more southern, temperate populations of both *D. simulans* and *D. melanogaster*. Rezende *et al.* (2011) remain critical of this justification, as it ignores the role of behavioural responses of adults in mediating thermal stress in nature. This is clearly true [as do all assays for upper thermal tolerance currently employed, including those advocated by Rezende *et al.* (2011) and Santos *et al.* (2011)]; however, we feel that the ramping assay of 0.06 °C min^{-1} is still justified, particularly since recent work (Overgaard *et al.*, 2012) indicates firstly that neither starvation nor desiccation stress confound estimates of upper thermal limits using ramping rates $\geq$ 0.06 °Cmin^{-1}, and secondly that the starting assumptions used in the models of both Rezende *et al.* (2011) and Santos *et al.* (2011) are flawed. Finally, we have focussed on heat knockdown time as a measure of thermal tolerance in adults, as opposed to critical thermal maxima (CT_{max}) to ensure that the measures used have the same units and thus may reflect similar underlying responses to heat stress (Santos *et al.*, 2011).

Previous studies of thermal adaptation in *D. simulans* (Arthur *et al.*, 2008; Sarup *et al.*, 2009) found no evidence for clinal or altitudinal patterns in thermotolerance. However, they relied on static measures of thermotolerance – the ability to withstand a sudden increase in temperature (25–39 and 25–37.7 °C respectively) – and may have been limited by small sample sizes. The fact that parallel clinal patterns in static and ramping (0.06 °C min^{-1}) heat knockdown time have

been observed in *D. melanogaster* (Sgrò *et al.*, 2010) suggests that both measures provide similar insight into patterns of adaptive differences among populations from along a climatic gradient, in spite of the concerns raised about the methodologies and ecological relevance surrounding all measures (Rezende *et al.*, 2011; Santos *et al.*, 2011). These parallel clines may either be the result of independent selection acting on the different measures, or they may reflect a shared genetic basis to static and ramping measures of heat resistance in *D. melanogaster*. The extent to which these different measures of thermal tolerance might provide similar insight into adaptive divergence in other species where no linear clines in thermal tolerance have previously been detected, such as *D. simulans* (Arthur *et al.*, 2008; Sarup *et al.*, 2009), remains unknown. However, such studies are important if we are to broadly understand how different species respond to similar selection pressures imposed by climate.

Clinal patterns for thermotolerance may also be influenced by differences in hardening (the ability to increase thermotolerance in response to short-term – minutes or hours – exposure to sublethal temperature stress) or acclimation (long-term exposure to temperatures that fall within the viable temperature range prior to a nonlethal temperature stress) capacity (Hoffmann *et al.*, 2003). Both *D. melanogaster* and *D. simulans* have shown acclimation responses to heat and cold stress (Hoffmann & Watson, 1993), and heat tolerance after hardening has also been shown to vary clinally in *D. melanogaster* (Sgrò *et al.*, 2010). No such clines in adults after hardening were apparent for populations of *D. simulans* from an altitudinal gradient in the Canary Islands (Sarup *et al.*, 2009), and the extent to which acclimation or hardening responses contribute to differences in adult thermotolerance across populations of *D. simulans* from eastern Australia has not previously been examined.

Many organsims, particularly insects, have complex life cycles with different life stages that experience different habitats and microhabitats with different environmental conditions (Kingsolver *et al.*, 2011). Despite the potential importance of differential selection across life stages, most clinal studies have focussed on adult thermotolerance. However, *Drosophila* egg and larval stages of development are exposed to thermal stress in nature (Feder *et al.*, 1997) and selection for heat tolerance at these stages may be stronger than in adults because of the reduced ability of eggs and larvae to bahviourally avoid thermal extremes.

Three recent studies have explicitly examined climatic selection in larvae. Sarup *et al.* (2006) found significant clinal variation for larval heat shock survival in *D. buzzatii* from eastern Australia. However, no such clines were observed along an altitudinal transect in either *D. buzzati* or *D. simulans* from the Canary Islands (Sarup *et al.*, 2009). Sgrò *et al.* (2010) observed clinal variation in egg to adult viability in *D. melanogaster* from eastern Australia under heat stress conditions that were simlar to the clines in adult heat tolerance, suggesting that pre-adult life stages are also under thermal selection in nature. Whether these clinal patterns are due to independent selection across life stages or reflect correlated reponses to selection across life stages remains unclear. Krebs & Loeschcke (1995) found little evidence that heat resistance is correlated across egg, larval and pupal stages in *D. buzzatti* populations from different geographical origins, whereas selection experiments on adults and larvae of *D. buzzati* showed responses only in the life-stage selected (Loeschcke & Krebs, 1996).

Additionally, our understanding of population differences in hardening and acclimation responses for heat tolerance in pre-adult stages is also limited. Hardening treatments can increase heat tolerance in pre-adult stages, as demonstrated by Feder *et al.* (1996) who found an increase in survival at 36 °C in *D. melanogaster* larvae and pupae after hardening. Yet, Sarup *et al.* (2006, 2009) found no clinal variation in hardened larval heat tolerance in *D. buzzatii* from eastern Australia or the Canary Islands. Similarly, no cline with altitude was found for hardened larval tolerance in *D. simulans* in the latter study. Furthermore, the extent to which dynamic assays of heat tolerance might affect latitudinal patterns in thermal stress resistance in pre-adult stages has not been assessed.

Most clinal studies of quantitative traits, including heat tolerance, in *Drosophila* have been performed in species that contain cosmopolitan inversions that also show clinal patterns (reviewed in Hoffmann *et al.*, 2004; Arthur *et al.*, 2008). Inversions inhibit recombination in heterozygotes as crossovers between inverted and standard chromosomal arrangements produce unviable and unbalanced gametes (Hoffmann *et al.*, 2004). Due to the lack of crossover, genes found within and around inversions are locked-up within the inversions, allowing selection to stabilize locally adapted combinations of alleles (Hoffmann *et al.*, 2004; Kirkpatrick & Barton, 2006). The extent to which differences in clinal patterns in heat tolerance across taxa might result from indirect selection on cosmopolitan inversions, or differences in the assays used across the different life stages, remains unknown. *Drosophila simulans* does not contain cosmopolitan inversion polymorphisms, so allows the study of climatic selection on quantitative traits that are not confounded by associations with inversions (Arthur *et al.*, 2008).

Finally, population demographic processes such as drift, gene flow or colonization history may also influence climatic adaptation along a latitudinal cline. High levels of gene flow may decrease the ability of populations to adapt to local conditions by introducing locally maladaptive variants (Slatkin, 1987; Hoffmann & Blows, 1994; Kirkpatrick & Barton, 1997), although evidence is limited (but see Bridle *et al.*, 2009). Asymmetrical gene

flow may also influence the shape of a cline, as higher emigration from populations with larger population sizes may override selection and decrease differentiation in nearby populations (James *et al.*, 1995). Alternatively, restricted gene flow may reduce local population size, resulting in drift that may increase differences in resistance between populations (van Heerwaarden *et al.*, 2009). Fast-evolving neutral markers such as microsatellites may clarify whether demographic factors or selection are involved in population differentiation along clines by assessing patterns of gene flow (van Heerwaarden *et al.*, 2009).

Given the increasing need to understand evolutionary responses to a warming climate, further work examining the extent to which clinal studies can in fact be used to gain insight into adaptive responses to climatic selection across taxa are required. In this study, we asked whether the absence of genetic clines in thermal tolerance previously reported in *D. simulans* (Arthur *et al.*, 2008; Sarup *et al.*, 2009) can be explained by the use of static assays of heat tolerance or small sample sizes. We assessed heat tolerance as static basal and hardened and ramping (0.06 °C min^{-1}; Sgrò *et al.*, 2010) measures of heat knockdown time. Furthermore, we assessed both adult and larval stages of development. Importantly, we combined these clinal studies with an assessment of population structure using neutral genetic markers to examine the relative importance of selection and gene flow.

Materials and methods

Clinal study

Field collection

Populations of *Drosophila simulans* were collected from 16 latitudes along the east coast of Australia between April and May 2008 (Table S1). Populations were collected as close to sea level as possible to minimize altitudinal differences between populations. Twenty field inseminated females were collected from each site to establish iso-female lines. Two to three generations after collection, a mass-bred population from each latitude was founded with 20 males and 20 females from each of the 20 iso-female lines. The mass-bred populations were kept at 25 °C under a 12 : 12 light/dark cycle in 2 × 250 mL bottles containing 60 mL of potato, yeast and sucrose media. Densities were approximately 300–350 flies per bottle to ensure a census population size of 600+ individuals.

Experimental protocol

A generation before each experiment below, each population was subjected to a series of short egg-laying periods (between 6 and 18 h) in 250-mL bottles containing 60 mL of potato, yeast and sucrose media. Larvae for the experimental generation were collected by placing a watch glass containing potato, yeast and sucrose media coloured with food dye, whose surface was covered in a live yeast paste to stimulate oviposition, atop an empty plastic bottle with flies from each population and allowing them to lay for 12 h at 25 °C.

Sixty first-instar larvae were picked into six 40-mL vials, each containing 8 mL of media for each population, and eclosed adults were collected from each vial. Only mated females (5–7 days post-eclosion) were tested. Flies were allowed to mate for 3–4 days and then separated under light CO_2 anaesthesia before being transferred to holding vials for 2 days at 25 °C prior to the assays described below.

Clinal heat tolerance assays

Basal and hardened static heat knockdown time. Basal and hardened static heat knockdown times were measured on fifty adult females per population and treatment. Adult flies were placed individually in 5 mL glass vials, exposed acutely to 38.5 °C by immersion in a preheated recirculating water bath. The hardening treatment involved exposure of flies to 35 °C for 30 min followed by recovery at 25 °C for 3 h prior to the knockdown assay being performed. Basal and hardened flies were tested simultaneously. Heat knockdown time was scored as the time taken for flies to be knocked down and immobilized. Treatments and populations were tested over eight runs spanning two consecutive days. Populations and treatments were randomized evenly across all runs. The static basal and hardened heat knockdown assays were performed after 11 generations of laboratory culture (F11).

Ramping (dynamic) heat knockdown time. Heat ramping knockdown resistance was measured in 50 adult females from each population. No hardening treatment was administered. Individual females were placed in 5-mL glass vials, which were submerged into a water bath heated to 28 °C. The temperature was increased gradually by 0.06 °C min^{-1} (following Terblanche *et al.*, 2007; Mitchell & Hoffmann, 2010) until all flies had been knocked down. This rate of increase was selected to simulate the rapid temperature increase during a hot day in south-eastern Australia (Fig. S1, http:www.bom.gov.au). A data logger (i-button) was submerged into the heated water bath (along with the flies) to record the temperature of the heated water bath throughout the experiment. Resistance was scored as the time taken for flies to be knocked down. Treatments and populations were tested over four runs spanning two consecutive days. Populations were randomized evenly across all runs. The heat ramping knockdown assay was performed at generation F12.

Larval heat survival

Basal and hardened static larval heat survival. A total of 15 replicate vials, each containing 20 second-instar

larvae, per treatment per population were tested in this assay. As with the adult survival assays described above, vials were capped, sealed with parafilm and inverted before being submerged into a preheated water bath. The hardening treatment involved exposure of larvae to 35 °C for 1 h followed by recovery at 25 °C for 1 h. Pilot studies showed that this treatment elicited a hardening response without causing elevated mortality. Unhardened and hardened larvae were then exposed to 38.5 °C for 1 h, then removed and placed at 25 °C to complete development. Survival was scored by recording all eclosed adults. Treatments and populations were tested over a total of six runs conducted over 2 days, with populations and treatments randomized evenly across all runs. The basal and hardened larval tolerance assay was completed simultaneously at generation F18.

Ramping (dynamic) larval heat survival. Fifteen replicate vials, each containing 20 second-instar larvae, per population were tested in the ramping assay. Vials were capped, sealed with parafilm and inverted before being submerged into the water bath. Similar to the adult knockdown ramping assay, vials were submerged into a water bath preheated to 28 °C and the temperature was then increased gradually by 0.06 °C min^{-1}, until the temperature of the bath reached 40 °C. Vials were left at 40 °C for 15 min, then removed and placed at 25 °C for larvae to complete development. This exposure was chosen based on pilot studies that indicated little or no mortality if larvae were removed before 40 °C, while few larvae survived beyond 40 °C. Data collected from rotting fruit in the field indicates that larvae developing in fruit exposed to full sun can experience temperature increases similar to those used here (Sgrò personal observation). Survival was scored by recording all of the eclosed adults. Treatments and populations were tested over two runs conducted on a single day, with populations and treatments randomized evenly across all runs. Ramping larval survival was assessed at generation F27.

Environmental variables

Data for 19 climatic variables were obtained from WORLDCLIM (version 1.3, http://www.worldclim.org) (Table S1). We focussed on both temperature means and extremes, as well as measures of climatic variability, since recent work suggests that climatic variability may be as, if not more, important in determining responses to climatic change than changes in the mean of climatic variables. These variables are derived from the monthly temperature and rainfall values (obtained by interpolation of climate station records) to generate more biologically meaningful variables. We used the program DIVA-GIS (version 7.2.3.1, http://diva-gis.org/) to extract bioclimatic variables (spatial resolution of 2.5 arc min) for each of the 16 collection sites for *D. simulans*.

Statistical analyses

Prior to analysis, the heat knockdown time data were log-transformed to achieve equal variances across populations. Arcsine square-root transformations were performed for the larval survival data because resistance was scored as a proportion. All statistical analyses were performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA). For static heat knockdown time and larval survival, two-way analyses of variance (ANOVAS) were used to test for differences between populations and the effect of hardening treatment. Population, treatment (basal or hardened) and run were treated as fixed effects. The interaction between population and treatment was also examined to test for differences in hardening response between populations. For the ramping heat knockdown and larval survival assays, one-way ANOVAS were used to test for differences among populations, with population and run as fixed effects.

Latitudinal patterns in all traits were analysed via regression analyses, with the means of each population as the data points. Each run was a complete randomized block containing all combinations of the populations being tested for that trait. We detected run effects (analyses described above), so prior to performing the regression analyses, individual values of each trait were corrected for run effects by multiplying each value by the ratio of the grand mean (over all runs for that trait) divided by the run mean (Sgrò *et al.*, 2010). This correction was performed for each trait separately. Although results were qualitatively the same when using uncorrected data, these corrected values were used in all regression analyses. We fitted quadratic and cubic regression terms to allow for nonlinear relationships with latitude and used log-likelihood ratio tests and AIC scores from generalized linear models to hierarchically test the significance of the linear, quadratic and cubic regression coefficients.

Finally, we explored whether environmental variables were significantly correlated with the thermotolerance clines along the latitudinal gradient examined. Principal component analysis (PCA) was used to reduce the 19 correlated temperature variables to principal components (PCs). The PCA was conducted on the correlation matrix. Multiple linear regressions, using forward selection, were performed to examine the effect of each the PCs on each of the traits examined.

Gene flow study

Field collection

A total of 330 *D. simulans* field males and females were collected via banana baits from 11 latitudes along the east coast of Australia (Table S2). Flies were collected between February and March in 2004. All

flies were stored in 100% ethanol at −20 °C prior to extraction. DNA was extracted using the chelex method.

Microsatellite genotyping and analysis

Fifteen female and 15 male *D. simulans* samples from each population were screened for seven polymorphic microsatellite markers (Hutter *et al.*, 1998). Polymerase chain reaction (PCR) of each locus was performed in 10-μL reactions containing 2 μL of template DNA, 1 μL of NEB, 2 mM dNTPs, 10 pmol μL^{-1} of each primer and (γ^{33}P)-ATP end-labelled forward primer. PCR cycling conditions were as described in Hutter *et al.* (1998). Amplified fragments were separated through a 5% denaturing polyacrylamide gel at 65 W for up to 2 h depending on fragment length. Dried gels were exposed for 8–24 h to auto-radiography film. Allele sizes were determined by comparison with λgt11 ladders (fmol DNA Cycle Sequencing System, Promega, Madison, WI, USA).

General measures of genetic variation, such as heterozygosity and number of alleles, were calculated with Genetic Analysis in Excel (GENALEX) 6.1 (Peakall & Smouse, 2006). GENEPOP 4.0.7 (Rousset, 2008) was used to examine whether there was linkage disequilibrium between loci and whether loci deviated from Hardy–Weinberg equilibrium (HWE). Micro-checker (Van Oosterhout *et al.*, 2004) was used to determine whether departures from HWE were due to genotyping errors such as stuttering, the presence of null alleles, short allele dominance or deviations from panmixia.

Genetic population structure was investigated through an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992), which partitions variation among and within populations. The AMOVA was performed in ARLEQUIN 3.1 (Excoffier *et al.*, 2005) with pairwise F_{ST} as the distance measure. Levels of population differentiation (F_{ST}) were examined overall and for pairs of populations using FSTAT 2.9.3 (Goudet, 1995). The significance of pairwise F_{ST} values was tested by permuting genotypes among populations (10 000 times), as this method does not rely on Hardy–Weinberg assumptions (Goudet, 1995). The significance of pairwise F_{ST} values was corrected for multiple comparisons using Bonferroni (Sokal & Rohlf, 1995). D_{est}, which is an estimator of actual differentiation, unbiased by levels of heterozygosity and gene diversity within demes (Jost, 2008), as well as G_{ST_est} and G'_{ST_est} were estimated across all populations and for each pair of populations using SMOGD (Software for the measurement of Genetic Diversity) (Crawford, 2010). Mantel tests (implemented in GENALEX (Peakall & Smouse, 2006) using pairwise F_{ST} values obtained from FSTAT 2.9.3 (Goudet, 1995), which were converted to Slatkins Linerized F_{ST} values (Rousset, 1997) as well as D_{est} estimates from SMOGD (Crawford, 2010), were undertaken to examine whether there was an association between genetic and geographical distance. A Bayesian clustering analysis, implemented in STRUCTURE v.2.3.3 (Pritchard *et al.*, 2000), was also used to infer genetic structure. Ten replicate runs were performed for values K = 1–11, where K is the number of independent genetic clusters. A burn-in length of 100 000 followed by 500 000 iterations was used for each simulation using an admixture model and uncorrelated allele frequencies among populations. The optimal number of clusters for our data was estimated by examining log-likelihood given the number of clusters [ln$P(X|K)$] (Pritchard *et al.*, 2000) and by examining the second-order rate of change of ΔK (Evanno *et al.*, 2005) using the program Structure Harvester (Earl & Holdt, 2012).

To investigate whether there was asymmetrical gene flow between populations, we used the software package MIGRATE version 3.0 (Beerli & Felsenstein, 2001) to estimate the effective number of migrants (4 *Nm*, where N is the effective population size and m is the migration rate) entering and leaving each population per generation. We relied on default search settings, using the microsatellite stepwise mutation model and running Migrate three times to verify the consistency of our results. Gene flow was considered asymmetrical if 95% confidence intervals comparing gene flow to and from a pair of populations did not overlap.

Results

Clinal study

Adult heat tolerance assays

Static heat knockdown time: basal and hardened. A two-way ANOVA showed significant differences between populations for static basal and hardened heat knockdown time (Table 1). The effect of run was significant. The hardening treatment had a significant effect, resulting in an increase in heat knockdown time (Table 1; also see Fig. 1), and there was no significant population by treatment interaction, indicating that the effect of hardening treatment did not differ among populations (Table 1).

Regression analyses revealed significant linear, quadratic and cubic components to the relationship between both basal and hardened static heat knockdown time and latitude (Table 2; Fig. 1); heat knockdown time increased from low to mid-latitudes, then decreased from mid- to high latitudes, with another increase at the two highest latitudes. Fitting linear, quadratic and cubic terms significantly improved the fit based on LRTs and AIC scores (not shown).

Ramping (dynamic) heat knockdown time and temperature. One-way ANOVA showed significant differences among populations for ramping heat knockdown

Table 1 One- and two-way analyses of variance (ANOVAS) testing for differences among populations for adult and larval heat tolerance.

Trait	Source	d.f.	SS	*F*	*P*-value
Adult static heat knockdown time (min)	Population	15	1.360	6.994	< 0.001
	Run	7	1.465	16.144	< 0.001
	Treatment	1	3.005	231.803	< 0.001
	Population × Treatment	15	0.319	1.642	0.057
	Error	1476	18.305		
Adult ramping (0.06 °C min^{-1}) heat knockdown time (min)	Population	15	0.003	5.107	< 0.001
	Run	3	0.007	57.466	< 0.001
	Error	754	0.029		
Larval static heat survival	Population	15	1.255	4.641	< 0.001
	Run	1	0.051	2.826	0.093
	Treatment	1	35.214	1952.840	< 0.001
	Population × Treatment	15	0.614	2.272	0.004
	Error	438	7.898		
Larval ramping (0.06 °C min^{-1}) heat survival	Population	15	1.694	7.052	< 0.001
	Error	223	3.570		

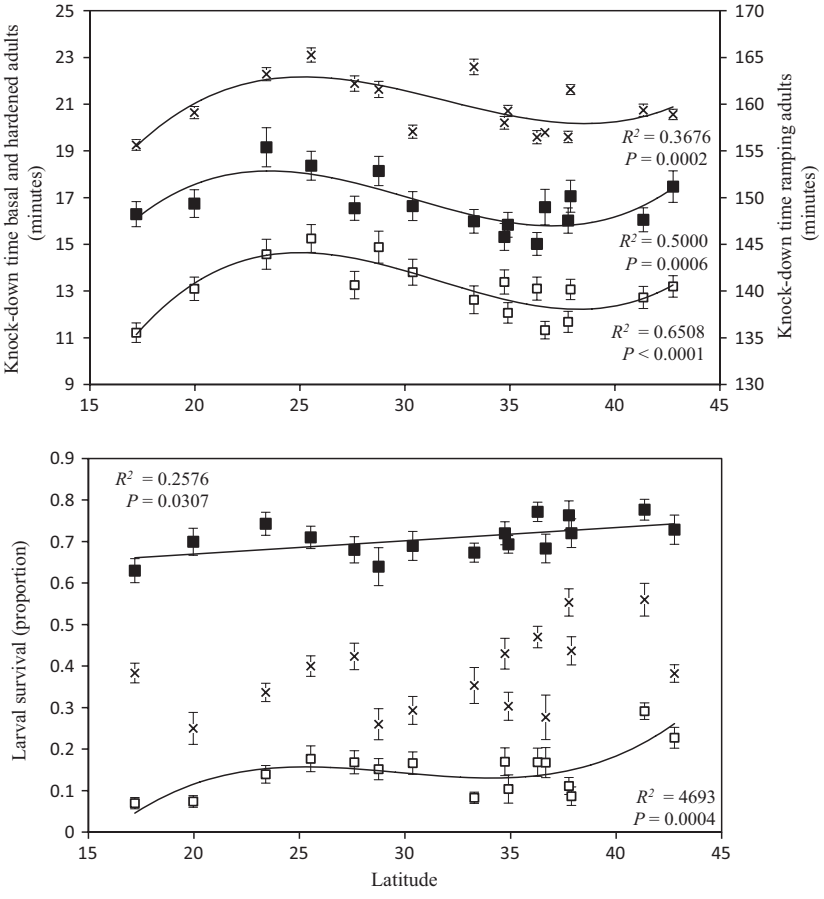

Fig. 1 Association between latitude and static and ramping adult heat knockdown time (top), and larval survival after a static and ramping heat stress (bottom) of *Drosophila simulans* populations originating from different latitudes along the east coast of Australia. Open squares represent basal treatments, closed squares represent heat hardened treatments and crosses represent heat ramping treatments. Linear, quadratic and cubic approximations and R^2 values are estimated by least-squares regression. Significance of linear, quadratic and cubic terms was estimated by likelihood ratio tests and AIC scores using generalized linear models. *P*-values correspond to the significance of the best-fit model based on likelihood ratio tests of a single intercept and zero slope.

temperature (CT_{max}) and knockdown time when temperature was increased at a rate of 0.06 °C min^{-1} (Table 1, Fig. 1). Once again, regression analyses revealed significant linear, quadratic and cubic components to the relationship between ramping heat knockdown time and latitude (Table 2; Fig. 1), and fitting

Table 2 Linear regression analyses testing for associations between latitude and adult and larval heat tolerance traits. Linear, quadratic and cubic associations are shown. Approximations (± standard deviation) and *F* ratio (degrees of freedom), *P*-value and adjusted R^2 of overall model estimated using least-squared means regressions are also shown

	Linear component		Quadratic component		Cubic component		Overall model		
Trait	*b* ± SD	*P*	*b* ± SD	*P*	*b* ± SD	*P*	*F* ratio (d.f.)	*P*	Adj. R^2
Basal static heat knockdown time	5.979 ± 1.190	0.0003	−0.3198 ± 0.041	0.0004	0.002 ± 0.0005	0.0005	10.319 (2,13)	0.001	0.6508
Hardened static heat knockdown time	4.99 ± 1.352	0.003	−0.173 ± 0.046	0.003	0.0019 ± 0.0005	0.0027	6.003 (2,13)	0.009	0.5000
Ramping heat knockdown time (0.06 °C min^{-1})	0.622 ± 0.209	0.012	−0.020 ± 0.007	0.015	0.0002 ± 7.86 × 10^{-5}	0.020	3.907 (2,13)	0.037	0.3676
Basal larval heat survival	0.206 ± 0.074	0.016	−0.007 ± 0.003	0.016	7.97 × 10^{-5} ± 2.77 × 10^{-5}	0.014	5.421 (2,13)	0.014	0.4693
Hardened larval heat survival	0.003 ± 0.001	0.026					6.204 (1,14)	0.026	0.2576
Ramping larval survival (0.06 °C min^{-1} from 28 to 40 °C)	0.005 ± 0.003	0.073					3.764 (1,14)	0.073	0.1556

linear, quadratic and cubic terms significantly improved the fit based on LRTs and AIC scores (not shown).

Larval heat tolerance assays

Basal and hardened static heat survival. A two-way ANOVA showed significant differences among populations for larval survival after heat stress (Table 1). The hardening treatment also had a significant effect, but a significant population by treatment was observed (Table 1, Fig. 1). Regression analyses revealed significant linear, quadratic and cubic components to the relationship between basal static heat survival and latitude, and fitting linear, quadratic and cubic terms significantly improved the fit based on LRTs and AIC scores (not shown). However, only the linear term was significant for hardened static heat survival (Table 2, Fig. 1).

Ramping (dynamic) larval heat survival. One-way ANOVA showed significant differences among populations for larval survival after a ramping heat stress (Table 1). Larval survival after a ramping heat stress increased linearly with latitude, although this relationship was marginally nonsignificant (Table 2, Fig. 1).

Clinal variation in traits and climate Pcs

The PCA revealed four PCs that accounted for over 96% of variance in the climatic variables (PC_1 = 52.729%, PC_2 = 26.832%, PC_3 = 10.018% and PC_4 = 7.300%, Table S3). Annual, maximum and minimum temperature, as well as rainfall contributed positively to PC_1, whereas climatic variability factors including diurnal range, annual temperature range and seasonality contributed negatively to PC_1 (Table S3). Diurnal and annual temperature range loaded positively onto PC_2, with negative contributions from precipitation (Table S3). Multiple regressions were performed between the PCs and each trait. Multiple regression analyses revealed that PC_2 was significantly positively associated with adult basal (adj. R^2 = 0.346, standardized partial regression coefficient = 0.0.624, t = 2.987, P = 0.010), hardened (adj. R^2 = 0.328, standardized partial regression coefficient: 0.611, t = 2.887, P = 0.012) and ramping (adj. R^2 = 0.225, standardized partial regression coefficient = 0.526, t = 2.313, P = 0.036) heat knockdown time. PC_1 was significantly negatively associated with larval basal (adj. R^2 = 0.237, standardized partial regression coefficient = −0.536, t = −2.377, P = 0.032) and hardened (adj. R^2 = 0.401, standardized partial regression coefficient = −0.664, t = −3.321, P = 0.005) heat survival. These results suggest that both precipitation in the drier months and variability in temperature play an important role in shaping the clinal patterns in adult heat knockdown time, whereas average and maximum temperature and precipitation during wetter months, as well as climatic variability, play an import role in larval heat survival along the latitudinal gradient examined here. No PCs were associated with ramping larval heat survival.

Gene flow analysis

All populations showed high levels of variation (Table S2). Allelic richness ranged from 5.20 to 7.43, with a mean of 6.37. Expected heterozygosity ranged from 0.670 to 0.745, with an average of 0.716 across all populations. Tests for linkage disequilibrium between all pairs of loci across populations found no significant linkage disequilibrium between loci. Significant departures [after Bonferroni correction (α = 0.004)] from HWE were observed for eight of the 11 populations (Table S2). In a total of 77 tests for each locus and population, after Bonferroni correction (α = 0.0006), significant departures from HWE, in the direction of het-

erozygote deficiency, were observed in seven cases (Tuncurry: D6a; Sydney: D10 and D119; Maryborough: D10; Red Rock: D119; Cairns: D119). In the populations and loci where departures from HWE were detected, Microchecker (Van Oosterhout *et al.*, 2004) found a significant frequency of null alleles. The presence of null alleles can lead to overestimates in the frequencies of other alleles and potentially influence the results of population structure analyses. To prevent such bias, we conducted all analyses twice: once using all seven loci and once excluding loci D10 and D119, which appeared to have the highest frequency of null alleles and deviations from HWE. For all analyses using F_{ST}, we also analysed the data with values corrected by FreeNA (Chapuis & Estoup, 2007). FreeNA first estimates null and visible allele frequencies for each locus and population, then allocates a single allele size not present in the original dataset to each null allele, then adjusts genotype frequencies based on the previous estimations of allele frequencies, and finally, calculates Weir's (1996) F_{ST} restricting the computation to visible states. In all cases, results were qualitatively identical; thus, we report results for the original full seven-loci analysis.

Overall, an AMOVA showed that most of the genetic variation at the micosatellite loci was attributed to differences among individuals within populations (96.69%), with only a small (but significant) proportion of genetic variation due to differences among populations (3.31%, $P < 0.001$). When only the mainland populations were considered, slightly less variation was detected among populations (3.00%, $P < 0.001$).

Overall, F_{ST} values were low, but significant (0.035; 95% CIs, 0.015–0.059) further suggesting little genetic differentiation among populations of *D. simulans* along the east coast of Australia. Averaged across all loci and populations, D_{est} (0.166; 95% CIs, 0.127–0.210) was higher than F_{ST}. Estimates of G_{ST_est} and G'_{ST_est} were 0.049 (95% CIs, 0.035–0.067) and 0.204 (95% CIs, 0.155–0.259), respectively. Population pairwise comparisons of F_{ST} ranged from 0.0017 to 0.0837, but no comparisons were significant after correction for multiple comparisons (Table S4). Mantel tests found a significant positive correlation between both Slatkin's linearized F_{ST} (Mantel $P = 0.007$; $R^2 = 0.170$) and D_{est} (Mantel $P = 0.002$; $R^2 = 0.224$) against the natural log of geographical distance (Fig. 2). Evaluation of $\ln P(X|K)$ and ΔK for different values of K supported the $K = 4$ model for our data (Evanno *et al.*, 2005). Proportion of membership was low within populations [average $Q = 0.249$ (range: 0.138–0.440)], suggesting little differentiation between populations, which is reflected in Fig. S2. No asymmetrical gene flow was detected between any populations (Table S5).

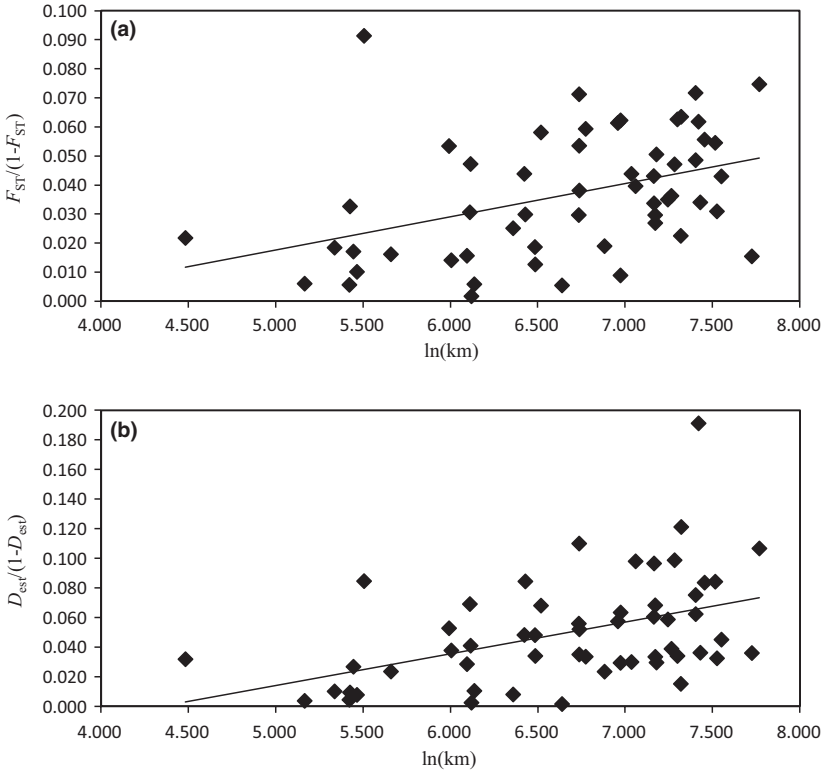

Fig. 2 Correlation between genetic and geographical distance between collection sites for microsatellites (a) ($F_{ST}/(1 - F_{ST})$; (b) ($D_{est}/(1 - D_{est})$).

Discussion

We found significant clinal variation in five of the heat tolerance traits examined, despite the significant levels of gene flow among the populations. We observed significant cubic clinal patterns for both static basal and hardened heat knockdown time and ramping heat knockdown time in adults, and static basal larval heat survival. In contrast, we found a significant positive linear clinal pattern for static hardened larval heat survival, whereas the positive linear cline for ramping larval heat survival was marginally nonsignificant.

The current results for adults contrast with earlier work looking at geographical variation in heat tolerance in *D. simulans* (Arthur *et al.*, 2008). This may reflect an increase in power to detect nonlinear clinal patterns in the current study, since Arthur *et al.* (2008) did not include any populations north of Maryborough (latitude 25°33′S), which is where we observed a decrease in heat knockdown resistance, that seems in part to drive the cubic association with latitude.

We did find significant associations between all three measure of adult heat knockdown time and climatic variables encompassing temperature variability and levels of precipitation during drier months. These associations suggest that the nonlinear clines in adult heat knockdown time are due at least in part to evolutionary response to climatic selection. Nonlinear clinal patterns have previously been reported for body size in *D. melanogaster* (James *et al.*, 1995), development time in *D. serrata* (Sgrò & Blows, 2003), heat tolerance in *D. buzzatii* (Sarup *et al.*, 2009) and cold tolerance in *D. simulans* from eastern Australia (Arthur *et al.*, 2008), suggesting either that nonlinear clines are influenced by nonlinear selection pressures (such as the climatic variables examined here) and/or that they reflect correlated responses to selection on other unmeasured traits. The cubic latitudinal patterns in adult heat tolerance reported here contrast with the significant (negative) linear associations with latitude recently reported for similar measures in *D. melanogaster* over the same latitudinal gradient in eastern Australia (Sgrò *et al.*, 2010). They also contrast with patterns observed for knockdown resistance in the cactophilic *D. buzzati* from along the east coast of Australia (Sarup *et al.*, 2006).

Although previous studies suggest that different assays of heat tolerance (e.g. surival vs. knockdown time) are genetically independent (Hoffmann *et al.*, 1997; Folk *et al.*, 2006), direct estimates of the additive genetic correlation between static and ramping measures of heat knockdown tolerance are lacking. Thus, we do not know the extent to which the cubic clines described above are the result of direct or correlated responses to selection. Interestingly, Mitchell & Hoffmann (2010) found that the additive genetic variance and narrow sense heritablity for ramping heat knockdown time in a northern and southern population of *D. melanogaster* from the east coast of Australia were not signficantly different from zero, compared to the higher and significant estimates for static heat knockdown time. This suggests (albeit indirectly) that these traits do not share a genetic basis in this species, and that there is limited evolutionary potential for insects to evolve in response to selection imposed by gradual increases in temerperature. Their results seem to be at odds with the parallel linear clines in female heat tolerance reported for *D. melanogaster* populations from eastern Australia (Sgrò *et al.*, 2010). However, Mitchell & Hoffmann (2010) did detect a positive interspecific correlation between static and ramping measures of heat tolerance, which suggests that these two measures may share a genetic basis. Quantitative genetic experiments estimating the additive genetic variance for, and additive genetic covariances between, these different measures of heat tolerance are required to determine whether these apparently different measures of heat tolerance are in fact genetically independent of each other, or whether they share a genetic basis and thus provide similar insight into the evolution of upper thermal limits.

Although we also found a significant nonlinear cline for static basal larval heat survival which parallels those found in the adults, we found a significant, positive latitudinal cline for static hardened larval survival. Larvae from more southern, temperate populations seem to be more resistant to heat stress than their tropical counterparts when exposed to sudden temperature following a hardening treatment. Separate heat selection on adults and larvae in *D. buzzati* (Loeschcke & Krebs, 1996) showed responses only in the life-stage selected, which suggests that levels of heat resistance are specific to the life cycle stage being examined. However, our data suggest that larval and adult stages show similar responses to climatic selection pressures along the cline examined, or that they share a genetic basis, at least for static basal larval survival. The linear cline in hardened larval survival suggests that this trait may be genetically independent of both basal and ramping larval survival. The significant association between static hardened larval survival and the climatic variables representing precipitation during wetter months, temperature, and climatic variability suggests that the observed clinal pattern is due at least in part to an evolutionary response to climatic selection. Population differences or altitudinal patterns in basal or hardened larval heat survival in *D. simulans* have not previously been reported (Sarup *et al.*, 2009).

We used the same test temperature and ramping rates to measure both adult heat knockdown time and larval heat survival, although the hardening treatments varied across the life stages. Although we used the same ramping rate increase of 0.06 °C min^{-1} in both adults and larvae, the maximum temperature reached in the larval assay was 40 °C for 15 min, whereas all

adults had succumbed to heat stress by 39.03 °C. These differences may have contributed to the different patterns observed for hardened responses to static heat stress and ramping across the life stages.

Although they did not directly examine larval thermal tolerance, Sgrò *et al.* (2010) observed a quadratic cline in *D. melanogaster* egg to adult viability under hotter developmental temperatures, with viability increasing from the tropics to mid-latitudes and then decreasing at the higher latitudes. This pattern also differs from the larval clinal patterns reported here. These differences suggest that larval stages of development may experience different selection pressures in the two species. It is also possible that larval survival measured at the second-instar stage as in the current study may not reflect egg to adult viability under heat stress. Previous work suggests that the relative levels of resistance to heat stress among populations changes with the different life stages examined (Krebs & Loeschcke, 1995).

The different patterns of heat tolerance in the adults and larvae of *D. simulans* and *D. melanogaster* from the same latitudinal gradient cannot be explained by asymmetrical gene flow or strong population genetic differentiation. We found high (symmetrical) gene flow and low levels of population differentiation in *D. simulans* populations from along the east coast of Australia, which are similar to those reported for *D. melanogaster* from eastern Australia (Agis & Schlotterer, 2001; Kennington *et al.*, 2003). In addition, our estimate of F_{ST} is comparable to that reported for *D. simulans* populations from Europe and northern Africa (Schofl & Schlotterer, 2006). Although we found a weak, but significant correlation between geographical and genetic distance in *D. simulans*, a similarly weak but significant correlation has been reported for *D. melanogaster* and *D. serrata* populations from the east coast of Australia (Kennington *et al.*, 2003; Frentiu & Chenoweth, 2009). Finally, linear clines for body size and starvation resistance observed in populations of both *D. simulans* and *D. melanogaster* from eastern Australia (James *et al.*, 1995; Arthur *et al.*, 2008) further support the conclusion that gene flow and population history cannot account for the nonlinear clines reported here.

Other than associations with climatic variables that themselves show nonlinear associations with latitude, it is not clear how selection has generated these varying clines in heat tolerance in *D. melanogaster* and *D. simulans* when they share a similarly cosmopolitan distribution. It may be the result of indirect effects of selection on cosmopolitan inversions, because one major difference between *D. melanogaster* and *D. simulans* is the presence of cosmopolitan chromosome inversions that vary with latitude in *D. melanogaster* (Arthur *et al.*, 2008). The cosmopolitan inversion *In(3R)Payne* has been shown to be associated with adult heat tolerance in Australian populations of *D. melanogaster* (Weeks *et al.*, 2002; Anderson *et al.*, 2003). Clinal patterns in heat tolerance in *D. melanogaster* may be driven by clinal selection on inversions rather than selelection on genes for heat tolerance themselves. It is also possible that these differences may reflect differences in heat avoidance strategies or behaviours (Prince & Parsons, 1977; Kawanishi & Watanabe, 1978) or differences in plasticity between the two species (David *et al.*, 2004) that have not been directly examined here.

Finally, it has been argued that estimates of upper thermal limits obtained using slower ramping rates such as those used in this study will be confounded by the simultaneous effects of resource depletion through desiccation and starvation and hardening/acclimation on organisms (Rezende *et al.*, 2011; Santos *et al.*, 2011). However, recent work (Overgaard *et al.*, 2012) suggests that such concerns will not have confounded the results described here. Furthermore, the parallel clines in the adult traits suggest that the static and dynamic assays used here provide similar insight into the extent of climatic adaptation in *D. simulans*. Multivariate quantitative genetic studies, within and across life stages, are required to determine whether these different measures of heat tolerance reflect a shared genetic basis. Such studies will also directly address recent concerns (Rezende *et al.*, 2011; Santos *et al.*, 2011) that the assays utilized in this and recent (Sgrò *et al.*, 2010) studies do not accurately describe adaptive differences or adaptive capacity in heat tolerance among populations.

Conclusions

In spite of high levels of gene flow, we found significant latitudinal variation for adult and larval heat tolerance in *D. simulans* from eastern Australia. However, these patterns were complex and differed across life stages. This indicates that it is essential that we consider multiple life stages in our efforts to understand the ecological and evolutionary consequences of ongoing warming. We also confirmed that *D. melanogaster* and *D. simulans* show very different patterns of local adaptation over a similar geographical range. This suggests that even closely related species, with very similar distributions and population genetic structure, may show different evolutionary responses to selection imposed by climatic changes, further complicating our efforts to understand climatic adaptation across taxa.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Site locations and climatic data[$] for populations of *Drosophila simulans* used clinal analysis of thermo-tolerance.

Table S2 Site locations and populations statistics for *Drosophila simulans* analysed with microsatellite markers.

Table S3 Principal Components analysis on temperature and variability data (see Table S1 for codes). Percentages for each PC shown in parenthesis.

Table S4 Pairwise F_{ST} (above diagonal) and D_{EST} (below diagonal) values.
Table S5 Estimates of gene flow (θ) between populations (latitude below in brackets) of *Drosophila simulans*. The range estimates given below each value are the 95% confidence limits.
Figure S1 Comparison of ramping temperature increase at a ramping rate of 0.06 °C min^{-1}(open squares) with actual increases in mean shaded temperature between the 22nd and 24th of January 2011 in Melbourne south-eastern Victoria Australia (solid circles), (www.bom.gov.au).
Figure S2 Summary plot of the proportional membership for each individual in each of four genetic clusters (K) for $K = 4$.

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