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No patterns in thermal plasticity along a latitudinal gradient in *Drosophila simulans* from eastern Australia

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Abstract

Phenotypic plasticity may be an important initial mechanism to counter environmental change, yet we know relatively little about the evolution of plasticity in nature. Species with widespread distributions are expected to have evolved higher levels of plasticity compared with those with more restricted, tropical distributions. At the intraspecific level, temperate populations are expected to have evolved higher levels of plasticity than their tropical counterparts. However, empirical support for these expectations is limited. In addition, no studies have comprehensively examined the evolution of thermal plasticity across life stages. Using populations of *Drosophila simulans* collected from a latitudinal cline spanning the entire east coast of Australia, we assessed thermal plasticity, measured as hardening capacity (the difference between basal and hardened thermal tolerance) for multiple measures of heat and cold tolerance across both adult and larval stages of development. This allowed us to explicitly ask whether the evolution of thermal plasticity is favoured in more variable, temperate environments. We found no relationship between thermal plasticity and latitude, providing little support for the hypothesis that temperate populations have evolved higher levels of thermal plasticity than their tropical counterparts. With the exception of adult heat survival, we also found no association between plasticity and ten climatic variables, indicating that the evolution of thermal plasticity is not easily predicted by the type of environment that a particular population occupies. We discuss these results in the context of the role of plasticity in a warming climate.

Introduction

As evidence for unprecedented global climate change increases, the need to understand how organisms respond to changes in climate, in particular warmer temperatures, has become increasingly important. Although behavioural thermoregulation (e.g. modifying daily activity patterns and/or selecting favourable microclimates) may ameliorate some of the effects of thermal stress (Dillon *et al.*, 2009; Huey & Pascual, 2009; Rego *et al.*, 2010), recent studies indicate that seasonal and latitudinal variation in climate is greater determinants of body temperature in ectotherms (Huey & Pascual, 2009) and that selection on tolerance to thermal extremes rather than behavioural responses is likely to be greater (Rego *et al.*, 2010). This suggests that the effectiveness of behavioural responses in ameliorating the effects of thermal stress may be limited and in small insects such as drosophilids physiological thermoregulation is virtually nonexistent (Stevenson, 1985). As such, the extent to which organisms are able to tolerate extreme temperatures through physiological and/or evolutionary mechanisms is likely to be vital for responding to ongoing climatic changes.

In ectotherms, responses to thermal extremes are complex and have been shown to be influenced substantially by inducible (plastic) responses (Brattstrom, 1970; Cossins & Bowler, 1987; Klok & Chown, 2003; Ayrinhac *et al.*, 2004; Hoffmann *et al.*, 2005; Chown &

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Terblanche, 2007; Angilletta, 2009; Hofmann & Todgham, 2010; Overgaard *et al.*, 2011a,b). Plastic thermal responses may be induced by short-term (minutes to hours) exposure to a sublethal temperature stress [hardening] or long-term (days to weeks) exposure to temperatures that fall within the viable temperature range [acclimation] (Hoffmann *et al.*, 2003; Colinet & Hoffmann, 2012). Although evolutionary responses are required for long-term survival to a changing climate, inducible stress responses may be an important initial mechanism to counter change (Cossins & Bowler, 1987; Calosi *et al.*, 2008; Terblanche *et al.*, 2008; Angilletta, 2009; Chown *et al.*, 2010; Hoffmann, 2010; Hofmann & Todgham, 2010; Hoffmann & Sgrò, 2011). As such, the survival and performance of ectotherms exposed to rapid thermal stresses are likely to depend on basal levels of tolerance, as well as plastic responses (Chown *et al.*, 2010; Hoffmann, 2010; Hoffmann & Sgrò, 2011). Nonetheless, our understanding of the evolution of thermal plasticity in natural populations remains limited (DeWitt & Scheiner, 2004; Brommer *et al.*, 2005; Nussey *et al.*, 2005, 2007; Pelletier *et al.*, 2007; Charmantier *et al.*, 2008; Husby *et al.*, 2010).

Theoretical studies suggest that environmental heterogeneity may be an important factor influencing the evolution of phenotypic plasticity in nature (Janzen, 1967; Levins, 1969; Moran, 1992; Sultan & Spencer, 2002; Ernande & Dieckmann, 2004; Ghalambor *et al.*, 2006; Chown & Terblanche, 2007). It has been hypothesized that tropical ectotherms should have lower plastic capacities than temperate species because they have evolved in environments that experience less daily and seasonal variation (Janzen, 1967; Levins, 1969; Ghalambor *et al.*, 2006; Chown & Terblanche, 2007; Tewksbury *et al.*, 2008; Angilletta, 2009). However, data from interspecific comparisons are scarce and equivocal (Brattstrom, 1970; Ghalambor *et al.*, 2006; Angilletta, 2009; Mitchell *et al.*, 2011; Overgaard *et al.*, 2011b). Overgaard *et al.* (2011b) observed a tendency for tropical species of *Drosophila* to show similar or slightly larger plastic responses to developmental heat and cold acclimation and heat hardening, compared with widespread *Drosophila* species, whereas widespread species showed a slightly larger cold-hardening response. Mitchell *et al.* (2011) observed only a weak trend for decreased plasticity of heat tolerance in tropically restricted *Drosophila* species when compared to widespread species. Finally, Brattstrom (1970) found no general latitudinal pattern when assessing acclimation responses in a wide range of Australian amphibians. Thus, it seems that despite strong theoretical support, clear differences in plasticity amongst tropically restricted and widespread species are not evident.

Although interspecific comparisons can reveal how past selection and phylogenetic history have shaped the evolution of thermal tolerance and plasticity, they are limited in the sense that they provide little information on the factors that might limit basal and plastic responses to thermal extremes between populations across a species' range. Importantly, comprehensive intraspecific studies of thermal plasticity for both upper and lower thermal limits, which explicitly study populations from a broad range of habitats that vary in levels of environmental variation and that also explicitly examine adult and pre-adult life stages, are lacking.

A powerful way of addressing this gap is to take advantage of naturally occurring clinal or geographic variation in plasticity. This is a particularly informative approach when used along environmental gradients where gene flow is high as it can provide evidence for natural selection and also provide insight into the principal evolutionary process underlying plasticity (Endler, 1977). Whereas this approach has been used to explore variation in plasticity across a small number of populations (Kipyatkov & Lopatina, 2002; Trotta *et al.*, 2006; Swindell *et al.*, 2007; Liefting & Ellers, 2008; Levine *et al.*, 2011; Fallis *et al.*, 2014), only a limited number of studies have examined clinal patterns in plasticity across a wide latitudinal range (James *et al.*, 1997; Azevedo *et al.*, 1998; Gilchrist & Huey, 2004; Liefting *et al.*, 2009; Sgrò *et al.*, 2010; van Heerwaarden & Sgrò, 2011) with mixed results. No clinal patterns in plasticity were evident for wing area (James *et al.*, 1997), wing centroid size, thorax length or wing-thorax size ratio (Azevedo *et al.*, 1998; van Heerwaarden & Sgrò, 2011) in *D. melanogaster* and *D. simulans* from eastern Australia. However, high latitude populations were marginally more plastic for female thorax length in *D. melanogaster* (James *et al.*, 1997) and wing loading in *D. subobscura* (Gilchrist & Huey, 2004). Liefting *et al.* (2009) found that development rate was more plastic in tropical compared with temperature populations, but body size showed higher levels of plasticity in the temperate populations compared with the tropical populations of *D. serrata* from eastern Australia. Importantly, only one study has explored whether the magnitude of plasticity in upper thermal limits varies across latitude (Sgrò *et al.*, 2010). Using a single measure of heat tolerance in adults, Sgrò *et al.* (2010) found that tropical populations of *D. melanogaster* were marginally more plastic than their temperature counterparts. No intraspecific studies have comprehensively examined thermal plasticity for lower thermal limits in adults or other life stages. Thus, there is mixed support for the hypothesis that higher levels of plasticity should evolve in more variable environments, with very little data specifically examining thermal plasticity for upper and lower thermal limits.

The overall aim of this study was to investigate patterns of thermal (heat and cold) plasticity in *D. simulans* populations from the east coast of Australia, examining both adults and larvae, which due to their differing ecologies are expected to experience different selection pressures (Kingsolver *et al.*, 2011). We have previously

detected clinal patterns in basal and hardened heat knock-down time in adults and heat survival in larvae of *D. simulans* from eastern Australia (van Heerwaarden *et al.*, 2012), but whether plasticity in these traits varies clinally has not been explored. In this study, we extend this work by including additional data on adult heat survival, adult chill coma recovery and adult cold survival to comprehensively investigate whether the extent of thermal plasticity [measured as hardening capacity (Kellett *et al.*, 2005)] is associated with latitude and/or measures of climatic variability and to explicitly test the theoretical prediction of lower levels of plasticity in tropical populations. We also examined whether there was any evidence for either shared mechanisms or trade-offs between the different measures of thermal plasticity by assessing correlations between hardening capacity for cold and heat tolerance in both adults and larvae.

Materials and methods

Field collection

Twenty field inseminated *Drosophila simulans* females were collected from 16 locations (latitudes) along the east coast of Australia between April and May 2008. The collection sites ranged from Sorell in southern Tasmania (latitude 42°46′S) to Gordonvale in Queensland (latitude 17°09′S) (Table S1). 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. Flies were always maintained at 25 °C under a 12 : 12-h light/dark cycle. The mass-bred populations were kept in 2 × 250-mL bottles containing 60 mL of potato, yeast and sucrose media, at an approximate density of 300–350 flies per bottle to ensure a census population size of 600 + individuals.

Preparation of experimental animals

Larval density was partially controlled for at least one generation prior to the experiments outlined below by subjecting each mass-bred population 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. Developmental density of the experimental flies was controlled by collecting first instar larvae. Thus, 50 breeding pairs were stimulated to lay eggs on spoons with media, and subsequently, first instar larvae were collected from the spoons and placed into empty 40-mL vials containing 8 mL of media. For each population, sixty first instar larvae were picked into six 40-mL vials containing 8 mL of media for each stress assay. Three to five days after eclosion, brief CO_2 anaesthesia was used to separate males and females and mated females (5–7 days post-eclosion) were subsequently tested for heat tolerance 2 days later (chill coma recovery 3 days later). We used several tolerance assays to assess the thermal tolerance of larvae or adults of *D. simulans* to ensure the generality of our findings to thermal plasticity more broadly. In all cases, we split animals from each population into two groups where one group was exposed to a pretreatment to induce a plastic response (hardening) and the other was acutely exposed to the thermal tolerance test (basal). Basal and hardened flies/larvae were tested simultaneously, with populations and treatments randomized evenly across runs.

Heat tolerance assays

Larval heat tolerance

In previous work, we examined clinal variation in basal and hardened larval heat survival, but not plasticity (van Heerwaarden *et al.*, 2012). In this study, we used the raw data from van Heerwaarden *et al.* (2012) to calculate hardening capacity (measured as the difference between basal and hardened heat survival) for each population. Briefly, for each population, 15 replicate vials containing 20 s instar larvae were used to assay basal and hardened larval heat tolerance. The hardening treatment involved exposing larvae to 35 °C for 1 h by immersing the vials in a preheated recirculating water bath and then allowing them to recover at 25 °C for 1 h. Basal and hardened larvae were then exposed to 38.5 °C for 1 h before they were returned to 25 °C, and tolerance was assayed from the number of flies that were able to complete development and emerge as adults. These treatments were chosen because pilot experiments indicated that the pretreatment (35 °C) resulted in a significantly positive hardening response while the stress treatment (38.5 °C) caused some, but not complete mortality such that the treatment allows for evaluation of latitudinal variation.

One potential complication in measuring hardening capacity for survival is that populations can only increase their survival to 100%, resulting in an upper limit to hardening responses. If the hardening treatment enhances survival to this level, comparing hardening capacity in a population with a basal larval survival of 50% that can potentially increase their heat survival by 50% to a population with a basal larval survival of 10% that can potentially increase their heat survival by 90% is erroneous. Because larval to adult survival is rarely 100%, even in untreated larvae, control vials for each population were also set up simultaneously (10 vials with 20 larvae per population) to confirm that the hardening treatment did not increase survival to the same level of unstressed larvae, which would indicate that the hardening treatment had reached an upper limit. The control, basal and hardened larval tolerance assays were completed simultaneously at generation F18 (18 generations in the laboratory), with the treated larvae tested over a total

of six runs (six randomized temporal blocks) conducted over 2 days.

Adult heat survival

The adult heat survival assay assessed 18 replicate vials of flies, per treatment per population. Each replicate consisted of 20 females (5–7 days post-eclosion) in vials with 5 mL of potato media. As described for the larval assays above, vials were capped, sealed with parafilm and placed inverted into the water bath. The hardening treatment involved exposing flies to 35 °C for 30 min followed by recovery at 25 °C for 3 h prior to the assay being performed. The assay involved exposing both hardened and unhardened flies to a heat stress of 38.5 °C for 33 min. Immediately after the stress, flies were returned to 25 °C. These treatments were chosen using the same criteria as for the larval heat treatments. Heat survival was scored 24 h after the test; all individuals displaying movement at this time were scored as alive. Treatments and populations were tested over six runs (six randomized temporal blocks) conducted over 2 days. Basal and hardened adult heat survival assays were tested at generation F15.

Adult heat knock-down

To calculate hardening capacity for heat knock-down in each population, we used raw data from van Heerwaarden *et al.* (2012), who examined clinal variation in basal and hardened adult heat knock-down time, but not plasticity. Briefly, basal and hardened heat knock-down times were measured on fifty females (5–7 days post-eclosion) per population and treatment by placing individual flies in 5-mL glass vials and then exposing them acutely to 38.5 °C. The hardening treatment involved exposing flies to 35 °C for 30 min and allowing them to recover at 25 °C for 3 h prior to performing the knock-down assay. The basal and hardened heat knock-down assays were performed over eight runs (eight randomized temporal blocks) on flies that had undergone 11 generations of laboratory culture (F11).

Cold tolerance assays

Chill coma recovery

Chill coma recovery time was assessed after 3 h at 0 °C in basal and cold hardened flies. Twenty to twenty-five females (5–7 days post-eclosion) were assayed per treatment, per population. Following established procedures (Hoffmann *et al.*, 2002; Arthur *et al.*, 2008), individual females were placed in sealed 5-mL glass vials and submerged into a 10% glycol solution cooled at a constant 0 °C. Chill coma recovery was scored as the time taken for flies to stand upright after being placed at 25 °C. Hardened flies were placed at 12 °C, 45 h prior to testing chill coma recovery (Anderson *et al.*, 2005), and were set up in testing vials at that temperature, before being placed directly at 0 °C for 3 h. Flies were scored by six observers in a single run, making sure that all populations and treatments were randomized between observers. These assays were performed at F23 of laboratory culture.

Adult cold survival

The adult cold survival assay involved simultaneously measuring survival in basal and cold hardened flies after 1 h at −6 °C and was performed at F8 of laboratory culture. The cold-hardening treatment consisted of a 2 h exposure to 4 °C followed by 2 h recovery at 24 °C. The survival assay was performed on 4–5-day-old flies using 5-mL glass vials with 10 flies (9-12) in each vial (10 vials per treatment, per population), and all flies (hardened and basal) of all populations were tested in the same run. Vials were capped, sealed with parafilm and submerged for 1 h in a 10% glycol solution cooled at a constant −6 °C. After cold exposure, flies were placed in 40-mL vials with food (placed on the side, so recovering flies did not get stuck in food) and allowed to recover at 25 °C. These treatments were chosen because pilot experiments indicated that they resulted in significantly positive hardening responses (i.e. an increase in survival with hardening) without resulting in total mortality or survival). Survival was scored after 7 h of recovery.

Statistical analyses

Prior to analysing the traits which were measured in different runs, individual values for each trait were standardized for run effects by multiplying each value by the ratio of the grand mean of all runs for that trait divided by the run mean (Sgrò *et al.*, 2010). This standardization was performed for each of these traits separately. As heat knock-down time was scored by two scorers during each run, we also corrected for scorer as above. These corrected values were subsequently used in all analyses and presentation of the data. All statistical analyses were performed using JMP for Windows version 4 (SAS Institute Inc., Cary, NC, USA), SPSS version 17 (SPSS Inc., Chicago, IL, USA) and R (R Core Team, 2014).

Clinal patterns in basal adult heat knock-down time and larval heat survival have already been presented (van Heerwaarden *et al.*, 2012), but here we analyse these data in the context of thermal plasticity. We first explored differences in plasticity (hardening responses) between populations by applying a two-way analysis of variance (ANOVA), with population and treatment (basal or hardened) as fixed effects. Prior to these analyses, the chill coma recovery time data were log-transformed to achieve equal variances across populations, whereas arcsine square-root transformations were performed for the survival data because resistance was scored as a proportion. To quantify the extent of thermal plasticity

(slope), we estimated absolute hardening capacity (AHC) for each trait and population as hardened stress tolerance minus basal stress tolerance, following Kellett *et al.* (2005). One-way ANOVAs were then used to test for differences in AHC for all traits among populations, with population as the fixed main effect. Latitudinal patterns in the extent of thermal plasticity were analysed via linear, quadratic and cubic regressions, with the mean AHC of each population regressed against latitude.

We also explored whether environmental variables representing climatic extremes and variability were significantly associated with patterns of plasticity along the latitudinal gradient examined. Data for ten measures of climatic variability/extremes (mean diurnal range, isothermality, temperature seasonality, maximum temperature of the warmest month, minimum temperature of the coldest month, temperature annual range, variance in monthly maximum temperature, variance in average temperature, variance in minimum temperature and precipitation) were obtained from WORLDCLIM (version 1.3, http://www.worldclim.org) (Table S1), by interpolation of climate station records from 1950 to 2000) 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. Multiple linear regressions, using the backward method, were performed to examine the effect of these measures of climate on AHC for each trait.

To determine the extent to which the different measures of hardening capacity were phenotypically correlated, Pearson's correlations were computed using the mean AHC of each population (Sokal & Rohlf, 1995).

Results

Heat tolerance assays

Larval heat survival

Clinal patterns in innate basal and hardened larval survival have previously been assessed (van Heerwaarden *et al.*, 2012); however, here we extend these analyses to explore clinal patterns in the extent of the plastic response (AHC). Briefly, across all the populations, basal larval heat survival ranged from 7% to 29% (average: 15%), whereas hardened larval heat survival ranged from 63% to 78% (average: 71%), which was lower than for the control lines (75–88%, average 79%). Thus, the hardening treatment improved larval heat survival, but not beyond the control vials. Overall, there were significant differences in heat tolerance among populations ($F_{15,438} = 4.641$, $P < 0.001$), a significant effect of hardening ($F_{1,438} = 1952.840$, $P < 0.001$) and a significant population by treatment interaction ($F_{15,438} = 2.272$, $P = 0.004$), suggesting that populations differed in their response to the hardening treatment.

Absolute hardening capacity (AHC) for larval survival after a heat shock in populations ranged from 48.5% to 65.2% (average: 55.8% difference between basal and hardened larval survival). There were significant differences among populations in AHC (Table 1), but there was no significant relationship between latitude and AHC (Table 2; Fig. 1a).

Adult heat survival

Basal adult survival ranged from 14% to 41% (average: 28%), whereas hardened adult survival ranged from 64% to 83% (average: 74%) such that AHC for adult survival after a heat shock ranged from 34% to 59% (average 47% difference between basal and hardened adult survival). A two-way ANOVA showed significant differences in heat tolerance among populations ($F_{15,534} = 5.368$, $P < 0.001$), a significant effect of hardening treatment ($F_{1,534} = 965.353$, $P < 0.001$) and a significant population by treatment interaction ($F_{15,534} = 2.189$, $P = 0.006$), suggesting that the hardening effect varied among populations. This was confirmed by one-way ANOVA that demonstrated a significant difference in AHC among populations (Table 1). However no significant relationship was found between AHC and latitude (Table 2; Fig. 1b).

Adult heat knock-down time

We have previously presented clinal patterns in innate basal and hardened adult heat knock-down time (van Heerwaarden *et al.*, 2012), and in this study, we extend these analyses to assess clinal patterns in the extent of the plastic response (AHC). Briefly, basal adult heat knock-down time ranged from 11.22 to 15.14 min (average: 13.03 min), whereas hardened adult heat knock-down time ranged from 14.87 to 19.10 min (average: 16.67 min). Overall, there were significant

Table 1 One-way analyses of variance (ANOVAs) testing for differences among populations for adult and larval hardening capacity. Traits measured are AHC (absolute hardening capacity) for larval heat survival, adult heat survival, adult heat knock-down time, adult cold survival and adult chill coma recovery time.

Trait	Source	d.f.	SS	*F*	*P* value
AHC larval heat survival	Population	15	0.649	1.773	0.040
	Error	214	5.226		
AHC adult heat survival	Population	15	1.492	1.931	0.021
	Error	264	13.599		
AHC adult heat knock-down	Population	15	621.069	1.422	0.130
	Error	685	19939.084		
AHC adult cold survival	Population	15	1.965	2.441	0.003
	Error	144	7.727		
AHC chill coma recovery	Population	15	1818.677	1.1750	0.289
	Error	342			

Table 2 Linear regression analyses testing for associations between latitude and thermal plasticity (AHC). Linear, quadratic and cubic associations shown. Approximations 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	Adj. R^2	*P*
AHC larval heat survival	−0.160 ± 0.093	0.110	0.006 ± 0.003	0.108	$-6.1 \times 10^{-6} \pm 3.48 \times 10^{-5}$	0.105	1.117	0.023	0.381
AHC adult heat survival	0.010 ± 0.131	0.943	−0.0006 ± 0.004	0.899	$7.51 \times 10^{-6} \pm 4.89 \times 10^{-5}$	0.880	0.735	< 0.001	0.551
AHC adult heat knock-down	−1.116 ± 1.653	0.512	0.029 ± 0.056	0.614	−0.0002 ± 0.0006	0.711	1.196	0.038	0.353
AHC adult cold survival	0.157 ± 0.213	0.476	−0.005 ± 0.007	0.467	$6.05 \times 10^{-5} \pm 8.00 \times 10^{-5}$	0.464	0.220	<0.001	0.881
AHC adult chill coma recovery	5.528 ± 3.791	0.170	−0.175 ± 0.129	0.201	0.002 ± 0.001	0.243	1.678	0.119	0.224

differences in heat tolerance among populations ($F_{15,1476} = 6.994$, $P < 0.001$), a significant effect of hardening ($F_{1,1476} = 231.803$, $P < 0.001$) and a nonsignificant population by treatment interaction ($F_{15,1476} = 1.642$, $P = 0.057$).

AHC for heat knock-down time ranged from 1.88 to 5.57 min improvement (average: 3.633 min difference between basal and hardened adult heat knock-down time). There were no significant differences among populations for AHC (Table 1) and no significant relationship between latitude and AHC (Table 2; Fig. 1c).

Cold tolerance assays

Adult cold survival

Basal adult cold survival ranged from 21% to 68% (average: 43%), whereas hardened adult cold survival ranged from 34% to 71% (average: 53%). A two-way ANOVA showed significant differences among populations for adult survival after a cold stress ($F_{15,288} = 5.605$, $P < 0.001$), a significant effect of hardening treatment ($F_{1,288} = 26.807$, $P < 0.001$) and a significant population by treatment interaction ($F_{15,288} = 2.388$, $P = 0.003$), suggesting that the effect of hardening varied among populations. AHC for adult cold survival in populations ranged from −5.6% to 44.9% (average: 10.0% difference between basal and hardened adult cold survival). There was a significant effect of population on AHC (Table 1), but no relationship with latitude was observed (Table 2; Fig. 1d).

Adult chill coma recovery

Basal adult chill coma recovery time ranged from 33.30 to 39.84 min (average: 36.38 min), whereas hardened adult chill coma recovery time was faster, ranging from 26.50 to 32.60 min (average: 29.80 min). A two-way ANOVA showed significant differences among populations for chill coma recovery time ($F_{15,728} = 2.267$, $P = 0.004$), a significant effect of hardening treatment ($F_{1,728} = 171.500$, $P < 0.001$) and a significant population by treatment interaction ($F_{15,728} = 1.705$, $P = 0.045$). AHC for adult chill coma recovery time ranged from 3.99 to 10.32 min improvement (average: 6.58 min difference between basal and hardened adult chill coma recovery time). There were no significant differences between populations in AHC (Table 1). No relationship between latitude and AHC was observed (Table 2; Fig. 1e).

Associations between absolute hardening capacity and climate variables

Multiple regression testing for an association between AHC and measures of climatic extremes/variability found a significant association only for AHC for adult heat survival (see below, other traits, data not shown). The best multiple predictor model ($F_{3,12} = 5.303$, $R^2 = 0.57$, $P = 0.015$, AIC = −42.391) included a combination of annual temperature range (slope = $5.841 \times 10^{-3} \pm 1.813 \times 10^{-3}$, $P = 0.007$), variance in monthly average temperature (slope = $-3.143 \times 10^{-4} \pm 1.346 \times 10^{-4}$, $P = 0.038$) and annual precipitation (slope = $6.894 \times 10^{-5} \pm 2.292 \times 10^{-5}$, $P = 0.007$).

Correlation analysis

To investigate whether hardening capacity across the different traits or life stages shared similar underlying mechanisms or might be constrained by trade-offs between the different measures of thermal plasticity, we performed a full factorial correlation analysis across all traits using population means. A significant correlation between AHC for adult cold and adult heat survival was observed (Table 3), but this was no longer significant after correcting for multiple comparisons (Bonferroni, $\alpha = 0.005$). No other significant correlations were observed between hardening capacity across the different thermal tolerance measures, including across life stage (Table 3).

Discussion

Recent papers have highlighted the importance of phenotypic plasticity for mediating rapid changes in climate (reviewed in Chevin *et al.*, 2010; Hoffmann & Sgrò,

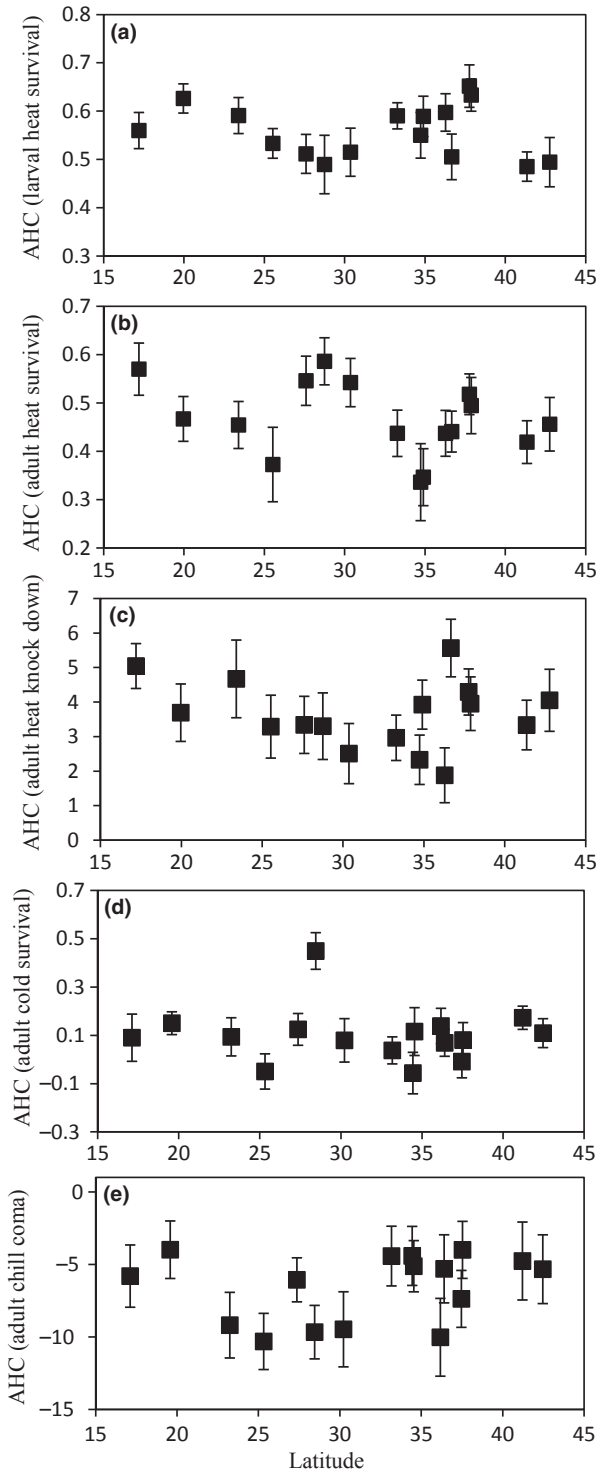

Fig. 1 Association between latitude and absolute hardening capacity (AHC) for (a) larval heat survival; (b) adult heat survival; (c) adult heat knock-down time; (d) adult cold survival; and (e) adult chill coma recovery. Hardening/acclimation treatments are described in the methods.

2011; Reed *et al.*, 2011). Consistent with past studies (e.g. Levins, 1969; Hoffmann & Watson, 1993; Krebs & Loeschcke, 1994; Bubliy *et al.*, 2002; Kellett *et al.*, 2005; Basson *et al.*, 2012), we found strong hardening responses for both upper and lower thermal limits. This pattern was evident across different thermal tolerance assays and across multiple life stages. With the exception of adult heat knock-down and chill coma recovery time, we found significant differences in absolute hardening capacity (AHC) between populations, suggesting that the extent of thermal plasticity genetically differs across populations. This result contrasts with several other studies which failed to detect any divergence in thermal plastic responses among a smaller number (2–8) of *D. melanogaster* populations from different geographical locations (Hoffmann & Watson, 1993; Bubliy *et al.*, 2002; Hoffmann *et al.*, 2005; but see Fallis *et al.*, 2014), but is concordant with studies that have found differences in thermal plasticity among a larger number (17–24) of populations of *D. melanogaster* (Ayrinhac *et al.*, 2004; Sgrò *et al.*, 2010; Overgaard *et al.*, 2011a).

Although a number of theoretical studies have hypothesized that tropical populations should exhibit lower levels of plasticity because they experience more stable environments (Janzen, 1967; Levins, 1969; Ghalambor *et al.*, 2006; Chown & Terblanche, 2007; Tewksbury *et al.*, 2008; Angilletta, 2009), we found no evidence to support this. We found no associations between latitude and hardening capacity for any of the thermal traits examined. With the exception of hardening capacity for adult heat survival, we also found no association between plasticity and climatic variables representing thermal extremes and variability, suggesting that the evolution of thermal phenotypic plasticity is not easily predicted from the type of environment that a particular species or population occupies. The climatic variables that could significantly explain some of the variation in hardening capacity for adult heat survival included a combination of annual temperature range, variance in monthly average temperature and annual precipitation, further supporting our conclusion that the evolution of thermal plasticity is complex. The absence of latitudinal patterns for thermal plasticity in *D. simulans* is in contrast to Sgrò *et al.*, 2010; who found hardening capacity for adult heat knock-down in *D. melanogaster* increased towards the tropics, but is consistent with a number of interspecific studies showing that temperate species do not show higher levels of thermal plasticity (Brattstrom, 1970; Overgaard *et al.*, 2011b).

It is not clear why theoretical predictions of lower levels of thermal plasticity in tropical populations are not observed for thermal tolerance in *D. simulans*. One possibility is that our measures of thermal plasticity using laboratory-based assays do not reflect plastic responses under selection in nature. Adult field release

Table 3 Pearson's correlation coefficients testing for correlations between the different measures of thermal hardening capacity.

Trait	AHC adult heat KD	AHC adult heat survival	AHC larval heat survival	AHC chill coma recovery
AHC adult heat survival	0.203 $P = 0.450$			
AHC larval heat survival	0.052 $P = 0.848$	−0.086 $P = 0.751$		
AHC chill coma recovery	0.256 $P = 0.338$	−0.226 $P = 0.399$	0.155 $P = 0.567$	
AHC adult cold survival	−0.023 $P = 0.932$	0.506 $P = 0.046$*	−0.339 $P = 0.200$	−0.186 $P = 0.491$

*Not significant after Bonferroni correction ($\alpha = 0.005$).

experiments have assessed the fitness of acclimated flies in nature (Loeschcke & Hoffmann, 2007; Kristensen *et al.*, 2008), finding higher capture rates (relative to untreated control flies) in heat hardened and cold acclimated flies under hot and cold conditions, respectively, in nature (Loeschcke & Hoffmann, 2007; Kristensen *et al.*, 2008) suggesting that these treatments have fitness benefits in nature. However, capture rates in cold acclimated and heat hardened flies were much lower than untreated flies under warm and cold field conditions, respectively, suggesting that cold acclimation and heat hardening might have a fitness cost under hot and cold field temperatures, respectively (Loeschcke & Hoffmann, 2007; Kristensen *et al.*, 2008). Fitness costs associated with the evolution of plasticity have frequently been proposed to limit the evolution of plasticity in natural populations (Lynch & Gabriel, 1987; Van Tienderen, 1991; Gilchrist, 1995; DeWitt *et al.*, 1998; Scheiner & Berrigan, 1998; Relyea, 2002; van Kleunen & Fischer, 2007; Callahan *et al.*, 2008; Van Buskirk & Steiner, 2009; Auld *et al.*, 2010; Basson *et al.*, 2012) and may also be influence the evolution of plasticity in tropical and temperate populations.

An absence of clinal patterns in plasticity may also be driven by low levels of additive genetic variance for thermal plasticity. Differences in plasticity (AHC) among populations for all measures of thermal tolerance besides heat knock-down and chill coma recovery indicate genetic variation for thermal plasticity, but additive genetic variance was not measured. While Fallis *et al.* (2014) observed significant genetic variation for thermal plasticity in chill coma recovery time in three of six populations of *D. melanogaster* from South America using an iso-female line approach, to our knowledge, no studies have directly measured additive genetic variance for thermal tolerance plasticity. Estimates of genetic variance for body size plasticity in *D. melanogaster* suggest that additive genetic variance for plasticity can be low (Scheiner, 1993). Whether this extends to other traits is not known.

Another possibility is that selection on innate thermal tolerance may be stronger than selection on plasticity. No clinal patterns were evident for basal or hardened adult heat survival, cold survival and chill coma recovery time (this study, data not shown), but significant latitudinal patterns for basal and hardened adult heat knock-down time and larval heat survival (van Heerwaarden *et al.*, 2012) suggest that these traits are under natural selection. However, as we do not know the strength of selection on either innate thermal tolerance or plasticity, it is not clear whether selection on innate thermal tolerance is influencing selection for thermal plasticity in the populations of *D. simulans* examined here. Selection on innate vs. plastic thermal resistance will also depend on the extent to which innate stress resistance and plasticity share underlying genetic mechanisms. Whether plasticity and the mean trait value in each environment are genetically independent has been the focus of much debate (e.g. Falconer, 1952; Via & Lande, 1985; Scheiner, 1993; Schlichting & Pigliucci, 1993, 1995; Via *et al.*, 1995; de Jong, 2005) and it remains unclear whether phenotypic plasticity is a function of differential expression of the same genes under different environments (Falconer, 1952; Via & Lande, 1985, 1987) or due to genes that determine the magnitude of responses to environmental effects which interact with genes that determine the average expression of the character (Lynch & Gabriel, 1987; Scheiner & Lyman, 1989; Scheiner, 1993; Schlichting & Pigliucci, 1993, 1995), or a combination of these factors.

It has been suggested that thermal plasticity, measured as the capacity to harden or acclimate, may be constrained by basal thermal tolerance (Stillman, 2003) and that this may limit the evolution of basal thermal tolerance and thermal plasticity in nature. Interspecific studies have provided conflicting evidence for a negative association between hardening capacity and thermal tolerance. Whereas Stillman (2003) found that porcelain crab species with higher levels of basal thermal tolerance showed the lowest acclimation capacity, positive associations between basal heat tolerance and hardening capacity have been reported in interspecific studies of *Drosophila* (Kellett *et al.*, 2005; Kristensen *et al.*, 2011; Mitchell *et al.*, 2011; Nyamukondiwa *et al.*, 2011) and diving beetles (Calosi *et al.*, 2008), although

a negative correlation between basal cold tolerance and hardening capacity was observed in the interspecific study of *Drosophila* by Nyamukondiwa *et al.* (2011).

Assessing the relationship between plasticity and the trait itself is, however, empirically problematic when hardening capacity is used to measure the extent of plasticity, as in the present study. Previous studies have regressed the magnitude of the plastic effects (hardening capacity) onto basal tolerance to test for a relationship between the two (e.g. Kellett *et al.*, 2005; Nyamukondiwa *et al.*, 2011). However, as hardening capacity is derived by taking the difference between basal and hardened tolerance, it is not clear to us whether regressing it back on to basal tolerance is a statistically valid approach (Kelly & Price, 2005). Inherently, populations can increase their hardening capacity through increases in hardened thermal tolerance, decreases in basal thermal tolerance or by both increases in hardened and decreases in basal thermal tolerance. Consequently, if hardening capacity is constrained by basal thermal tolerance, we would expect to see increases in hardening capacity to occur only as a consequence of decreases in basal thermal tolerance. Therefore, rather than exclusively examining the association between hardening capacity and basal resistance, as carried out in most other studies, we examined how changes in both basal and hardened thermal tolerance are driving differences in hardening capacity. Although a significant negative relationship between basal thermal tolerance and hardening capacity is observed for four of the five traits (Fig. 2), a significant negative association between hardening capacity and basal thermal tolerance, with no association with hardened thermal tolerance, was found only for adult and larval heat survival (Fig. 2a,b). Nonetheless, as these two traits are measures of survival, this finding may reflect a statistical limitation, rather than a true physiological limitation because survival can only increase to 100%. These results suggest that hardening capacity in *D. simulans* is not constrained by basal thermal tolerance. Thus, the lack of empirical support for theoretical predictions of higher thermal plasticity in temperate populations in *D. simulans* does not seem to be explained by a trade-off with between basal tolerance and hardening capacity. Quantitative genetic experiments assessing the genetic covariance between basal thermal resistance and thermal plasticity are required to preclude a fundamental evolutionary constraint.

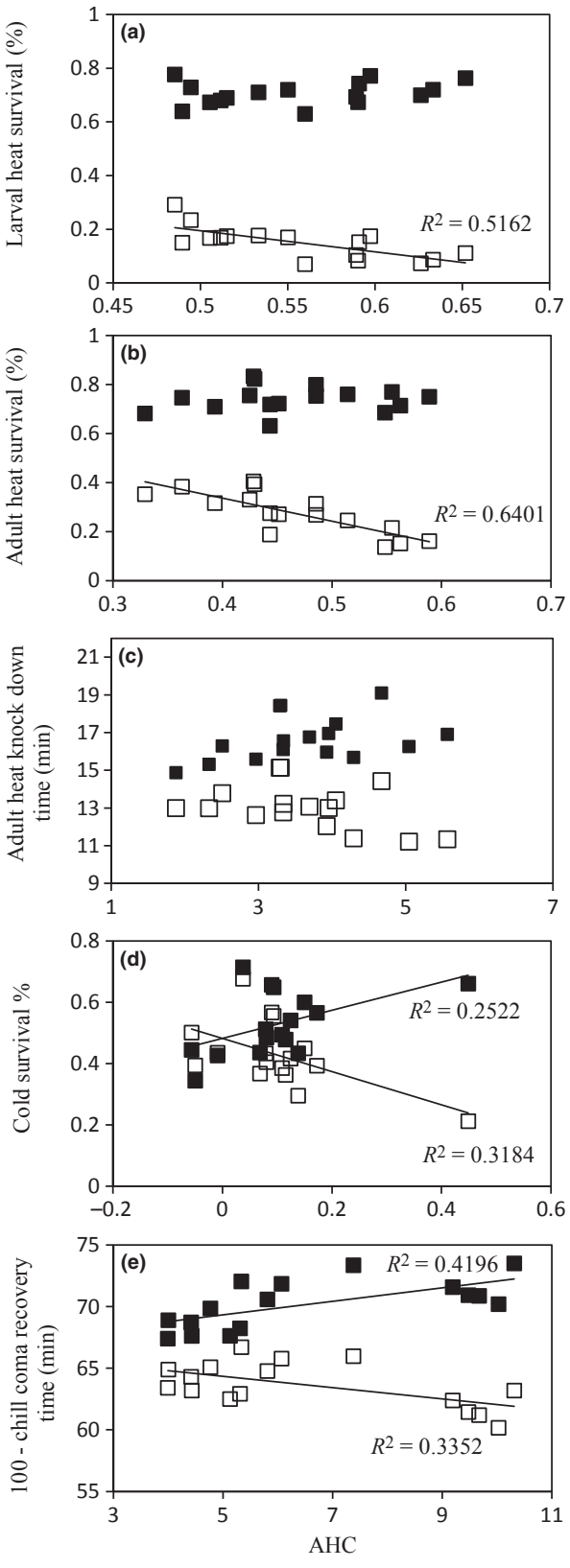

Fig. 2 Association between absolute hardening capacity (AHC) and basal (open squares) and hardened (solid squares) thermal tolerance for (a) larval heat survival; (b) adult heat survival; (c) adult heat knock-down; (d) adult cold survival; and (e) adult chill coma recovery time. Significant associations ($P < 0.05$) are plotted.

The extent to which plasticity in cold tolerance may trade off with plasticity in heat tolerance has also been the focus of recent interspecific studies. Calosi *et al.* (2008) observed a significant positive correlation in the ability to acclimate to heat and cold across 13 species of European diving beetles. In contrast, Nyamukondiwa *et al.* (2011) found no correlation between rapid cold hardening and rapid heat hardening in different species of *Drosophila* suggesting that the physiological mechanisms underlying cold and heat hardening differ. Consistent with this latter result, we found no evidence to suggest that similar mechanisms or patterns of selection underlie hardening capacity for heat and cold tolerance at the intraspecific level. A lack of significant negative correlations between hardening capacity across the different stress traits also suggests that there are no trade-offs between plasticity for different thermal tolerance traits.

Numerous studies have suggested that the mechanisms underlying basal levels of thermal tolerance appear to differ depending on the type of assay method used and which life stage is examined (Hoffmann *et al.*, 1997; Addo-Bediako *et al.*, 2000; Rako & Hoffmann, 2006; Folk *et al.*, 2007; Chown *et al.*, 2009; Mitchell & Hoffmann, 2010). Metamorphic organisms have distinct life stages which may vary in size, morphology and physiology, as well as in which habitat they occupy, exposing them to different microclimates and selection pressures (Kingsolver *et al.*, 2011). As such, climatic selection is unlikely to act on only one life stage in metamorphic organisms and geographic patterns in thermal plasticity may differ across different life stages. However, little is known about how these differences in ontogeny might be reflected in differences in hardening capacity. Consistent with previous work on basal thermal tolerance (Hoffmann *et al.*, 1997; Addo-Bediako *et al.*, 2000; Rako & Hoffmann, 2006; Folk *et al.*, 2007), we found no significant correlations between thermal plasticity (hardening capacity) for the different measures of heat or cold tolerance, within or across life stages. This suggests that patterns of selection and/or the mechanisms underlying hardening capacity differ across different measures of thermal tolerance and across different life stages.

In conclusion, strong hardening responses across all measures of thermal tolerance and across life stages highlight the potential for phenotypic plasticity to play an important role in responses to current and future climatic changes. However, we found no evidence to support the hypothesis that temperate populations have evolved higher levels of thermal plasticity than their tropical counterparts. Furthermore, the lack of a significant association between plasticity and climatic variables for all but one measure of thermal resistance examined suggests that the evolution of thermal plasticity is not easily predicted from the environment. Our results provide no evidence to suggest that selection favours a general mechanism underling thermal plasticity in *D. simulans,* although quantitative genetic or gene expression studies would shed more light on this issue. The extent to which our results might be influenced by low genetic variation for thermal plasticity, costs of plasticity and/or negative genetic correlations between thermal plasticity and basal thermal tolerance is unknown, but should be the focus of future studies.

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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 in the clinal analysis of thermotolerance and thermal hardening capacity.

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