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Limited scope for plasticity to increase upper thermal limits

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Summary

1. Increases in average temperature and the frequency of extreme temperature events are likely to pose a major risk to species already close to their upper physiological thermal limits. The extent to which thermal phenotypic plasticity can buffer these changes and whether plasticity is constrained by basal tolerance levels remains unknown.

2. We examined the effect of developmental temperature under both constant and fluctuating thermal regimes (developmental acclimation), as well as short-term heat hardening, on upper thermal limits (CTmax) in a tropical and temperate population of *Drosophila melanogaster*.

3. We found evidence for thermal plasticity in response to both developmental acclimation and hardening treatments; CTmax increased at warmer developmental temperatures and with a prior heat hardening treatment. However, hardening and acclimation responses were small, improving CTmax by a maximum of 1·01 °C. These results imply that overheating risk will only be minimally reduced by plasticity.

4. We observed significant associations between developmental temperature and both basal CTmax and hardening capacity (a measure of the extent of the plastic response). Basal CTmax increased, while hardening capacity decreased, with increasing developmental acclimation temperature. This indicates that increases in basal heat resistance at warmer temperatures may come at the cost of a reduced capacity to harden.

5. While plasticity in CTmax is evident in both populations of *D. melanogaster* we studied, plastic increases in upper thermal limits, particularly at warmer temperatures, may not be sufficient to keep pace with temperature increases predicted under climate change.

Key-words: acclimation, climate change, CTmax, hardening, heat, reaction norm, thermal tolerance

Introduction

Temperature is an important factor determining the distribution and abundance of species (Cossins & Bowler 1987). Both average temperature and the frequency and intensity of extreme temperature events are projected to increase over the coming decades (Clusella-Trullas, Blackburn & Chown 2011; Diffenbaugh & Field 2013; IPCC 2013), imposing increasingly strong selection on many taxa. Climate change is therefore increasingly likely to affect species distribution and abundance (Burrows *et al.* 2012) and risk of extinction (Deutsch *et al.* 2008; Tewksbury, Huey & Deutsch 2008).

Ectotherms are particularly vulnerable to temperature changes because of the close association between environmental and body temperatures. Many ectotherm species are thought to already be living close to their upper physiological thermal limits (Addo-Bediako, Chown & Gaston 2000; Deutsch *et al.* 2008; Tewksbury, Huey & Deutsch 2008; Kellermann *et al.* 2012). The fact that variation in upper thermal limits is much lower than for lower thermal limits in many terrestrial ectotherms (Addo-Bediako, Chown & Gaston 2000; Huey *et al.* 2009; Sunday, Bates & Dulvy 2011; Kellermann *et al.* 2012; Araujo *et al.* 2013; Grigg & Buckley 2013) suggests that the scope for species to increase their heat resistance via evolutionary responses to increasing selection pressures may be low. Moreover, direct assessments of adaptive capacity for upper thermal limits suggest that responses to selection may be constrained by low levels of genetic variation (Baer & Travis 2000; Hammond & Hofmann 2010; Mitchell & Hoffmann 2010; Kelly, Sanford & Grosberg 2012; Kelly, Grosberg & Sanford 2013; Blackburn *et al.* 2014) and that

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responses to selection for increased heat resistance may be small and plateau rapidly (Hoffmann *et al.* 1997; Gilchrist & Huey 1999; Bubliy & Loeschcke 2005; Schou *et al.* 2014). While these studies suggest a limited capacity to evolve higher levels of heat tolerance in ectotherms, they ignore the extent to which phenotypic plasticity may increase thermal tolerance and mitigate the negative impacts of climate change.

Plastic increases in thermal tolerance can occur in response to both short-term (minutes to hours) sublethal hardening treatments and longer term (days to weeks) acclimation treatments that fall within a species viable temperature range (Cossins & Bowler 1987). Thus, by buffering organisms from rising temperatures, plasticity may play a crucial role in determining a species' sensitivity to climate change (Chevin, Lande & Mace 2010). Despite this, comprehensive tests of the extent to which plasticity in upper thermal tolerance can buffer organisms from rising temperatures under climate change are lacking.

Two main theories have been proposed to explain variation in thermal plasticity. The trade-off hypothesis predicts that selection for higher thermal tolerance will come at a cost to plasticity (Stillman 2003; Somero 2010; Gunderson & Stillman 2015). However, empirical support for this theory is equivocal (Cavicchi *et al.* 1995; Lerman & Feder 2001; Stillman 2003; Kellett, Hoffmann & McKechnie 2005; Calosi, Bilton & Spicer 2008; Mitchell, Sgrò & Hoffmann 2011; Castañeda, Rezende & Santos 2015; Magozzi & Calosi 2015). Stillman (2003) found that porcelain crab species living in habitats with the highest maximum temperatures showed the lowest acclimation capacity, consistent with predictions. In contrast, interspecific studies in *Drosophila* (Kellett, Hoffmann & McKechnie 2005; Mitchell, Sgrò & Hoffmann 2011; Nyamukondiwa *et al.* 2011) and diving beetles (Calosi, Bilton & Spicer 2008) found positive associations between basal heat tolerance and hardening capacity. However, these results may be confounded by the fact that these conclusions were based on direct comparisons between basal thermal resistance and hardening/acclimation capacity, which violate the assumption of statistical independence because hardening/acclimation capacity is calculated using basal resistance (Kelly & Price 2005). Finally, a recent meta-analysis across a wide range of ectothermic species found no association between basal heat tolerance and thermal plasticity (Gunderson & Stillman 2015).

The latitudinal, or seasonality, hypothesis predicts a pattern of increasing thermal plasticity with latitude (Janzen 1967). Thus, tropical species and populations from low latitudes should display lower levels of plasticity than their temperate (high latitude) counterparts because they have evolved in environments that experience less daily and seasonal variation (Janzen 1967; Levins 1969; Ghalambor *et al.* 2006; Chown & Terblanche 2007; Angilletta 2009). Once again, empirical support for this theory is equivocal (Hoffmann & Watson 1993; Calosi *et al.* 2010; Mitchell, Sgrò & Hoffmann 2011; Overgaard *et al.* 2011; van Heerwaarden *et al.* 2014; Gunderson & Stillman 2015). Interspecific studies reveal inconsistent or very weak and negative associations between latitude and thermal plasticity (Brattstrom 1970; Tsuji 1988; Calosi *et al.* 2010; Mitchell, Sgrò & Hoffmann 2011; Overgaard *et al.* 2011; Gunderson & Stillman 2015). Intraspecific studies have also produced mixed results (Hoffmann & Watson 1993; James, Azevedo & Partridge 1997; Azevedo *et al.* 1998; Gilchrist & Huey 2004; Sgrò *et al.* 2010; van Heerwaarden *et al.* 2014).

Although questions remain about the mechanisms that drive patterns of plasticity, studies suggest that plastic increases in CTmax can be quite small (e.g. Brett 1956; Kingsolver & Huey 1998; Kellett, Hoffmann & McKechnie 2005; Overgaard *et al.* 2011; Gunderson & Stillman 2015), indicating that overheating risk under climate change may be minimally reduced by thermal plasticity. Nonetheless, previous studies are limited in two ways. First, most examine a limited range (two–three) of temperature/acclimation treatments (e.g. Terblanche *et al.* 2006; Mitchell, Sgrò & Hoffmann 2011; Overgaard *et al.* 2011). Secondly, to our knowledge, none have considered the combined effects of short-term hardening and long-term acclimation under ecologically relevant developmental temperatures on CTmax, even though both will contribute to species responses to climate change (Sgrò, Terblanche & Hoffmann 2016).

In this study, we comprehensively assessed for the first time the extent to which plasticity can increase thermal tolerance by quantifying shifts in upper thermal limits (measured as CTmax) in response to both short-term hardening and longer term developmental acclimation in a tropical and temperate population of *Drosophila melanogaster*. We did so by assessing basal and hardened CTmax after development at six constant developmental acclimation temperatures spanning most of the viable thermal range of *D. melanogaster* (Economos & Lints 1986). We also examined the response of CTmax to fluctuating developmental acclimation temperatures that reflect the average range of temperatures encountered in the field in warmer (summer and spring) months in tropical and temperate regions of Australia that are both ecologically and biologically relevant to *D. melanogaster* (www.bom.gov.au). This allowed us to, first, explicitly quantify the extent to which developmental acclimation under constant or fluctuating thermal regimes, combined with short-term hardening, substantially shifts CTmax. Secondly, it allowed us to test the trade-off hypothesis by quantifying the extent to which plastic responses are limited by basal heat resistance or by development at higher temperatures. Thirdly, we were able to ask whether plastic responses differed across tropical and temperate populations in a direction consistent with the latitudinal or seasonality hypothesis.

Materials and methods

EXPERIMENTAL POPULATIONS

Populations of *D. melanogaster* were collected from a temperate (Melbourne, Victoria, 37·8136°S, 144·9631°E) and tropical

(Innisfail, north-eastern Queensland, 17·5236°S, 146·0292°E) location in April 2014. Populations along the east coast of Australia have been evolving in Australia for over 100 years and are an excellent model for understanding short-term evolutionary responses to climate (Hoffmann & Weeks 2007). Specifically, previous studies have shown clinal variation in a number of traits and genes (reviewed in Hoffmann & Weeks 2007), as well as significant genetic differentiation (Kennington, Gockel & Partridge 2003; Kolaczkowski *et al.* 2011) between tropical and temperate populations along the east coast of Australia, suggesting that these populations are phenotypically and genetically differentiated. Thirty single-field-inseminated females were collected from each location from banana baits and maintained in the laboratory as separate isofemale lines for two generations. Two generations after field collection, a mass-bred population was established for each location by pooling 10 virgin females and males from each isofemale line (totalling 600 flies per population) into two 250-mL bottles containing potato dextrose agar medium. For each successive generation, each mass-bred population was maintained at 25 °C under a 12:12 light: dark cycle at a census population size of approximately 1000 individuals across three 250-mL bottles containing potato dextrose agar medium. CTmax was assessed after 11 (constant) and 13 (fluctuating) generations of mass breeding.

DEVELOPMENTAL ACCLIMATION – CONSTANT AND FLUCTUATING DEVELOPMENTAL TEMPERATURES

CTmax was measured on basal and hardened adult female flies from both populations that had developed from egg to adults under six different constant temperature regimes: 16 °C, 18 °C, 20 °C, 25 °C, 28 °C and 30 °C; as well as four different fluctuating temperature regimes with different average daily means: 20 °C (15–25 °C), 23 °C (18–28 °C), 26 °C (21–31 °C) and 28 °C (23–33 °C) (see Table S1, Supporting information for more details). These thermal regimes were chosen because they represent the temperature range experienced in the field during the warmer (summer and spring) months in temperate and tropical Australia (www.bom.gov.au). Density was controlled by picking 40 eggs into 20 vials per thermal regime. As developmental time varies across different thermal regimes, egg picking was staggered so that adult flies would eclose from the different temperature regimes on the same day. The sexes were separated using CO_2 anaesthesia 2 days post-eclosion, and females were given 4 days to recover before hardening. All experimental females were therefore mated.

HEAT HARDENING TREATMENTS

The hardening treatment involved placing flies in groups of five in a sealed vial containing 3 mL of potato dextrose agar medium, which was inverted and completely submerged in a circulating water bath heated to constant 37 °C for 1 h (pilot data revealed that this treatment maximized the hardening response across both populations; Fig. S1). After hardening, flies were left to recover for a further 23 h at 25 °C.

ESTIMATING CTMAX

CTmax was assessed on basal and hardened flies simultaneously by placing individual flies in 10-mL dry vials sealed and submerging them in a water bath heated to 25 °C. The temperature was then gradually increased at a rate of 0·1 °C min^{-1}, and CTmax was scored as the time and temperature at which flies went into a heat coma (i.e. no movement). While the question of how best to study upper thermal limits has been the focus of recent debate (Rezende, Tejedo & Santos 2011; Santos, Castaneda & Rezende 2011; Terblanche *et al.* 2011; Overgaard, Kristensen & Sorensen 2012) and some theoretical studies have expressed concern that these dynamic assays may be confounded by desiccation and starvation resistance (Rezende, Tejedo & Santos 2011; Santos, Castaneda & Rezende 2011), recent studies in *D. melanogaster* indicate that such effects are negligible (Overgaard, Kristensen & Sorensen 2012; Hangartner & Hoffmann 2015). Furthermore, we have shown that estimates of adaptive capacity (additive genetic variation) using a dynamic ramping method contribute to the adaptive capacity of upper thermal limits in *Drosophila* (van Heerwaarden & Sgrò 2013). CTmax was assessed across six runs over 2 days (constant developmental regime flies) or across nine runs over 3 days (fluctuating developmental regime flies), with three different scorers/observers in a randomized block design on 7-day-old female flies.

ANALYSES

All analyses were conducted in SPSS 17.0. We used analyses of variance (ANOVAS) to assess the effect of population, developmental acclimation temperature, hardening and their interactions, as well as run and scorer, on CTmax. We consistently find that scorer (observer) influences estimates of CTmax; however, by including scorer as an effect in the model, we account for these effects, and in doing so obtain unbiased estimates of CTmax (van Heerwaarden & Sgrò 2013; van Heerwaarden *et al.* 2014). The effects of constant and fluctuating developmental acclimation temperatures were examined in separate analyses. All factors were designated as fixed effects. *Post hoc* analyses (Sidek) were used to examine which treatments were driving differences across developmental acclimation temperatures and the hardening treatment.

To quantify the level of thermal plasticity in response to hardening, we calculated hardening capacity as absolute hardening capacity: hardened CTmax – basal CTmax; and relative hardening capacity: (hardened CTmax – basal CTmax) / basal CTmax (Kellett, Hoffmann & McKechnie 2005; Sgrò *et al.* 2010). Absolute and relative hardening capacity were calculated individually for each population, at each developmental acclimation temperature. We then used two-way ANOVAS to examine the effect of developmental acclimation temperature and population, and their interaction, on absolute and relative hardening capacity. To examine the relationship between hardening capacity, basal CTmax and developmental temperature, we used linear regressions, with the mean relative hardening capacity and basal CTmax of each population regressed against developmental acclimation temperature. Linear regressions are appropriate because the overall slope is estimated across each stepwise temperature comparison, resulting in linear slopes.

To quantify the level of thermal plasticity in response to developmental acclimation and to compare the acclimation response to hardening capacity, we calculated developmental acclimation capacity. This was calculated as absolute acclimation capacity (CTmax at higher acclimation temperature – Ctmax at cooler acclimation temperature) and relative acclimation capacity ((CTmax at higher acclimation temperature – Ctmax at cooler acclimation temperature) / Ctmax at cooler acclimation temperature). Absolute and relative acclimation capacity were estimated across all stepwise temperature comparisons (e.g. constant 30 vs. constant 28; constant 28 vs. constant 25; fluctuating 28 vs. fluctuating 26; fluctuating 26 vs. fluctuating 23) and were calculated separately for each developmental regime treatment (constant/ fluctuating), population and hardening treatment (basal/ hardened).

Acclimation response ratio (ARR), a measure of the magnitude of difference between two developmental acclimation temperatures as a function of the degree of change, was calculated for basal and hardened treatments. ARR was calculated separately for each developmental regime treatment (constant/ fluctuating), population and hardening treatment (basal/ hardened) using the equation [(CTmax at temperature 1 (e.g. 18 °C) – CTmax at

temperature 2 (e.g. 16 °C)] / °C degree change between the two different temperatures (e.g. 2 °C) (Levins 1969; Kingsolver & Huey 1998; Gunderson & Stillman 2015). ARR was estimated across all stepwise temperature comparisons (e.g. constant 30 vs. constant 28; constant 28 vs. constant 25; fluctuating 28 vs. fluctuating 26; fluctuating 26 vs. fluctuating 23). ARR was then averaged across each of these temperature treatment comparisons to give an average ARR for each developmental regime treatment (constant/ fluctuating), population and hardening treatment (basal/ hardened). An ARR of 1 indicates a positive 1 °C shift in CTmax for each 1 °C increase in developmental temperature, suggesting complete compensation as developmental temperature increases. In contrast, an ARR of 0 indicates that CTmax is unaffected by developmental temperature.

To examine the maximum level of thermal plasticity in response to developmental acclimation and hardening, maximum plastic responses (hardened CTmax at the highest developmental temperature – basal CTmax at the lowest developmental temperature) were calculated for each population at both constant and fluctuating temperature environments.

Finally, to examine the capacity for acclimation responses for CTmax to buffer increases in temperature, we used basal ARR averaged across all temperatures for each population (mean basal CTmax ARR), to calculate how much thermal plasticity can reduce overheating risk (the difference between mean environmental temperature and CTmax) for a given change in developmental/ acclimation temperature under warming. This was calculated as follows: change in overheating risk basal = (Mean basal CTmax ARR − 1) × change in developmental temperature (Gunderson & Stillman 2015). We also examined the extent to which maximum plastic responses (i.e. hardening as well as acclimation responses) can further reduce overheating risk and compensate for increases in developmental/acclimation temperature under warming (maximum plastic response ratio). This was calculated for each stepwise temperature comparison as follows: hardened CTmax at temperature 1 (e.g. hardened CTmax at 18 °C) – basal CTmax at temperature 2 (e.g. basal CTmax at 16 °C) / °C degree change between the two different temperatures (e.g. 2 °C). These values were then averaged for each population and then used to calculate change in overheating risk incorporating hardening and acclimation responses using the following equation: (Maximum plastic response ratio − 1) × change in developmental temperature.

Results

CONSTANT DEVELOPMENTAL ACCLIMATION REGIME

When we examined the influence of developmental acclimation across six different constant temperatures on CTmax, we found that CTmax increased with increasing developmental temperature for both populations irrespective of hardening treatment, but these responses were small (<1 °C average change) (Fig. 1; Table 1). Overall, short-term hardening increased CTmax in both populations, but again these responses were small (<0·2 °C average change) (Fig. 1; Table 1). Innisfail had a higher CTmax than Melbourne, regardless of hardening treatment (Fig. 1). Population, developmental temperature, hardening treatment and run all had significant effects on CTmax (Table 2; Fig. 1). *Post hoc* analyses (Sidek) revealed a significant effect of heat hardening in the Melbourne population at constant 16 °C ($P < 0{\cdot}05$), and 26 °C ($P < 0{\cdot}05$) developmental temperatures (Fig. 1). For Innisfail, a significant effect of heat hardening on CTmax was observed in flies that had developed at 16 °C ($P < 0{\cdot}001$), 18 °C ($P < 0{\cdot}01$), 26 °C ($P < 0{\cdot}05$) and 28 °C ($P < 0{\cdot}05$) (Sidek *post hoc* test; Fig. 1).

FLUCTUATING DEVELOPMENTAL ACCLIMATION REGIME

Similar to the patterns observed under constant developmental temperatures, we found that CTmax increased with increasing developmental temperature for both

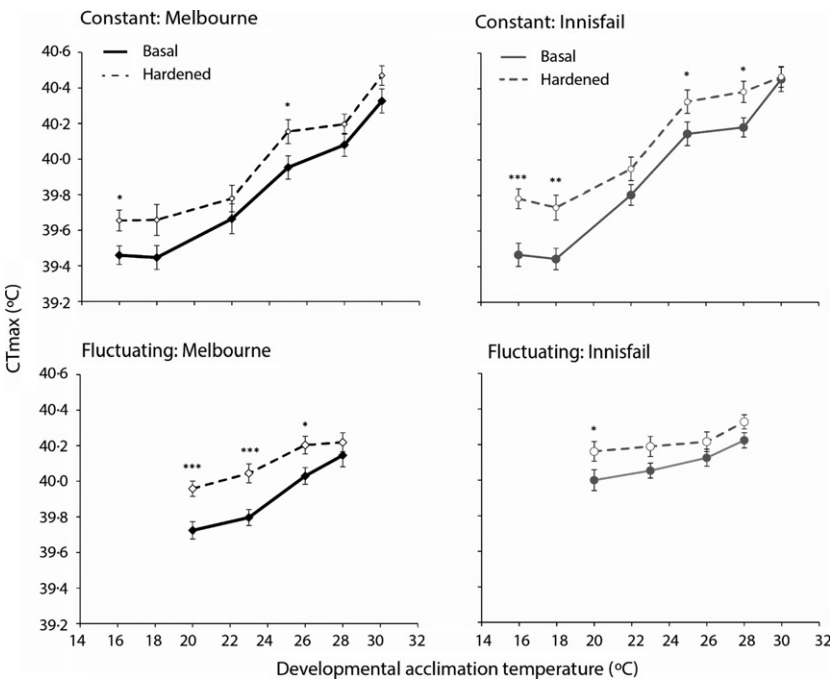

Fig. 1. Thermal reaction norm for basal (solid line) and heat hardened (37 °C for 1 h) (dashed line) CTmax for Melbourne (left) and Innisfail (right) across different constant (top) and average fluctuating (bottom) developmental acclimation temperatures. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$ indicates significant differences between basal and hardened CTmax within each developmental regime.

Table 1. Average differences in absolute and relative hardening capacity and absolute and relative acclimation capacity as well as the acclimation response ratio (ARR) across the different populations, developmental temperature regimes and hardening treatments (developmental acclimation only).

		Hardening capacity		Acclimation capacity (Basal)			Acclimation capacity (Hardened)		
Population	Temperature Regime	Absolute	Relative	Absolute	Relative	ARR	Absolute	Relative	ARR
Melbourne	Constant	0·164 °C	0·004 (0·4%)	0·867 °C	0·022 (2·2%)	0·062 °C	0·815 °C	0·022 (2·2%)	0·062 °C
	Fluctuating	0·186 °C	0·005 (0·5%)	0·422 °C	0·011 (1·1%)	0·053 °C	0·261 °C	0·007 (0·7%)	0·030 °C
Innisfail	Constant	0·191 °C	0·005 (0·5%)	0·987 °C	0·025 (2·5%)	0·068 °C	0·685 °C	0·017 (1·7%)	0·043 °C
	Fluctuating	0·124 °C	0·003 (0·3%)	0·224 °C	0·006 (0·6%)	0·030 °C	0·166 °C	0·004 (0·4%)	0·025 °C

Table 2. Analysis of variance examining differences in CTmax between populations, constant developmental temperatures and heat hardening treatments.

Source of variation	d.f.	MS	*F*	*P*
Population	1	2·396	30·466	<0·001
Acclimation temperature	5	14·299	181·832	<0·001
Hardening	1	4·301	54·694	<0·001
Run	8	4·222	53·682	<0·001
Scorer	2	0·233	2·961	0·052
Population × acclimation temperature	5	0·111	1·416	0·216
Population × hardening	1	0·001	0·007	0·934
Acclimation temperature × hardening	5	0·130	1·656	0·143
Population × acclimation temperature × hardening	5	0·068	0·863	0·506
Error	682	0·079		

Table 3. Analysis of variance examining differences in CTmax between populations, fluctuating developmental acclimation temperatures and heat hardening treatments.

Source of variation	d.f.	MS	*F*	*P*
Population	1	2·447	54·623	<0·001
Acclimation temperature	3	1·502	33·525	<0·001
Hardening	1	2·555	57·038	<0·001
Run	5	1·713	38·236	<0·001
Scorer	2	0·469	10·467	<0·001
Population × acclimation temperature	3	0·280	6·253	<0·001
Population × hardening	1	0·049	1·095	0·120
Acclimation temperature × hardening	3	0·057	1·263	0·287
Population × acclimation temperature × hardening	3	0·010	0·230	0·876
Error	410	0·045		

populations, regardless of hardening treatment, but these responses were small (average <0·5 °C) (Fig. 1; Table 1). Hardening treatment also increased CTmax, but again these effects were very small (average <0·2 °C), and were generally largest at the lower acclimation temperatures (Fig. 1; Table 1). Innisfail was generally more heat-resistant than Melbourne, especially in basal flies (Fig. 1). CTmax varied more across developmental temperature in Melbourne than in Innisfail, with a lower CTmax at lower developmental temperatures in Melbourne, irrespective of hardening (Fig. 1). A significant effect of population, developmental temperature, heat hardening, run and scorer, and a significant population-by-developmental temperature interaction were observed (Table 3; Fig. 1). Correcting for scorer by multiplying each value of CTmax by the ratio of the overall grand mean (over all scorers) divided by scorer mean (Sgrò *et al.* 2010) had no effect on the results (data not shown), so we retained scorer as a factor in the ANOVA for simplicity.

HARDENING CAPACITY

The absolute and relative differences in CTmax between basal and hardened flies were used to explore hardening responses across developmental temperature, treatment (constant vs. fluctuating) and population. The magnitude of these plastic responses (absolute and relative hardening capacity values) was small across both populations (Table 1). For absolute hardening capacity, the hardening response improved CTmax on average for Melbourne by 0·164 °C (from 39·822 to 39·986 °C) and 0·182 °C (from 39·924 to 40·107 °C) and Innisfail by 0·191 °C (from 39·916 to 40·107 °C) and 0·123 °C (40·101 to 40·224 °C) under constant and fluctuating developmental temperatures, respectively (Table 1). Similarly, relative hardening capacity was also extremely low (Fig. 2; Table 1), with hardening improving CTmax by only 0·4% to 0·5% in

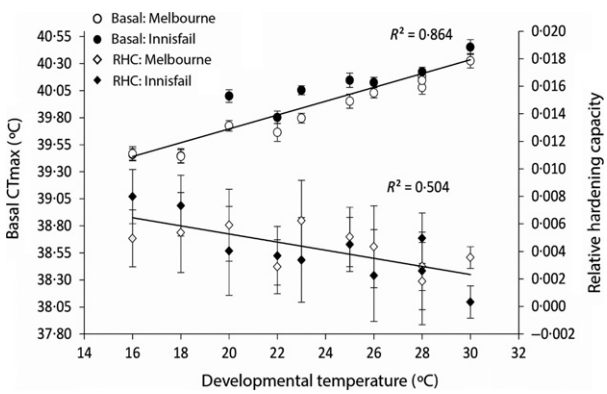

Fig. 2. Association between developmental acclimation temperature (constant and fluctuating) and relative hardening capacity (squares) and basal (circles) CTmax for Melbourne (open) and Innisfail (solid).

Melbourne flies, and 0·3% to 0·5% in Innisfail flies. Absolute and relative hardening capacity were similar in the tropical and temperate populations, and across the constant and fluctuating temperature regimes (Table 1). We found a significant effect of developmental temperature on absolute and relative hardening capacity, but no effect of population or treatment (fluctuating vs. constant) and no interactions between population and developmental temperature or population and treatment (constant vs. fluctuating) were found for either absolute or relative hardening capacity (Tables S2 and S3).

In the light of the non-significant population term noted above, we combined the data from Melbourne and Innisfail to explore the relationship between developmental temperate and hardening capacity in more detail. A significant positive association was observed between developmental temperature and basal CTmax ($F_{1,18}$ = 114·657, P < 0·001, b = 0·064, R^2 = 0·864; Fig. 2). In contrast, a significant negative association was found between developmental temperature (regardless of whether it was constant or fluctuating) and relative hardening capacity ($F_{1,18}$ = 18·261, P < 0·001, b = −0·00029, R^2 = 0·504), suggesting that hardening responses are lower at higher developmental temperatures (Fig. 2). These patterns were also present for absolute hardening capacity and when the populations were examined separately (data not shown). Taken together, these results suggest that hardening responses are constrained by higher levels of basal resistance at warmer developmental temperatures (Fig. 2).

ACCLIMATION CAPACITY

Acclimation responses were larger than hardening responses, but still extremely small (Table 1). Under the fluctuating regime, basal CTmax changed on average by 0·867 °C (Melbourne) and 0·987 °C (Innisfail), while under the constant regime basal CTmax changed by 0·422 °C (Melbourne) and 0·224 °C (Innisfail). Acclimation effects were even lower for hardened flies, changing basal CTmax by only 0·815 (Melbourne) and 0·685 °C (Innisfail) under the fluctuating regime and by 0·261 °C (Melbourne) and 0·166 °C (Innisfail) under the constant regime. Average values of relative acclimation capacity under the constant temperature treatments ranged from 1% to 2·5%, and 0·4% to 1·1% under the fluctuating temperature treatments (Table 1). Acclimation response ratios (ARR), which measure the change in CTmax as a function of change in temperature, were also extremely low, ranging from a 0·030 to 0·068 °C (average 0·053 °C) increase in CTmax per 1 °C increase in developmental temperature for basal CTmax and from 0·025 to 0·062 °C (average 0·040 °C) increase for hardened CTmax (Table 1).

MAXIMUM PLASTIC RESPONSE

Maximum plastic responses (hardened CTmax at the highest developmental temperature – basal CTmax at the lowest developmental temperature) were low: 0·495 °C (1·2%) and 0·328 °C (0·8%) for Melbourne and Innisfail, respectively, under fluctuating developmental temperatures and 1·01 °C (2·6%) and 1 °C (2·5%) under constant developmental temperatures.

When we explored the capacity for developmental acclimation and hardening to buffer increases in temperature, we found that plastic responses were unable to substantially compensate for increases in thermal overheating risk (Fig. 3), even when both hardening and acclimation responses (maximum plastic responses) were taken into account.

Discussion

Rises in average temperatures of between 2 and 4 °C have been predicted by the end of the century (IPCC

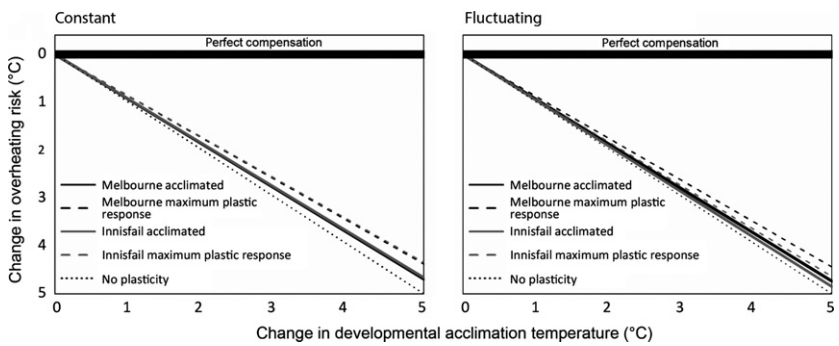

Fig. 3. Predicted changes in overheating risk (the difference between mean environmental temperature and CTmax) given changes in mean developmental acclimation temperature under constant (left) and fluctuating (right) developmental temperature regimes. The thin solid lines (see key) represent predicted changes in overheating risk based on mean CTmax ARR values calculated for each population considering only developmental acclimation responses (acclimated). The thin dashed lines represent predicted changes in overheating risk based on maximum plastic response that incorporates both acclimation and hardening effects (maximum plastic response). The fine dotted line shows predicted changes in overheating risk in the absence of any acclimation or hardening responses – without plasticity, overheating risk matches the increase in developmental temperature. The thick solid horizontal black line shows the predicted changes in overheating risk if plasticity (developmental acclimation and/ or hardening) perfectly compensates for changes in developmental acclimation temperature, resulting in no overheating risk. Modified from Gunderson & Stillman (2015).

2013), and these are likely to exceed current upper thermal limits for many species, particularly tropical and mid latitude species (Deutsch *et al*. 2008; Tewksbury, Huey & Deutsch 2008; Clusella-Trullas, Blackburn & Chown 2011; Kellermann *et al*. 2012). Past studies investigating thermal acclimation responses over a limited number (typically two or three) of temperatures have generally observed small plastic changes in CTmax (Brett 1956; Kingsolver & Huey 1998; Overgaard *et al*. 2011; Gunderson & Stillman 2015). However, none of these studies have considered the joint effects of acclimation and short-term hardening on plastic shifts in CTmax (but see Crill, Huey & Gilchrist 1996 for an examination of cross-generational effects on CTmax plasticity), despite the fact that both will likely contribute to thermal plasticity under climate change (Sgrò, Terblanche & Hoffmann 2016). We address these shortcomings by performing a comprehensive assessment of phenotypic plasticity for CTmax, simultaneously examining hardening and developmental acclimation responses across a range of both constant and fluctuating temperatures in a tropical and temperate population of *D. melanogaster*.

Consistent with previous studies (Kingsolver & Huey 1998; Overgaard *et al*. 2011; Gunderson & Stillman 2015), we observed only small shifts in CTmax (0·166–0·987 °C) in response to developmental acclimation temperature. Hardening responses were even smaller, increasing CTmax by only between 0·124 and 0·191 °C. The maximum plastic response, taking both developmental acclimation and hardening responses into consideration, increased CTmax by only 1·01 °C. Thus, developmental acclimation and rapid hardening failed to substantially shift CTmax. Given that a recent study found that adaptive genetic responses to increased temperature in *D. melanogaster* may increase upper thermal limits by only 0·5 °C (Hangartner & Hoffmann 2015; also see Morrison & Milkman 1978; Huey, Partridge & Fowler 1991; McColl, Hoffmann & McKechnie 1996), evolutionary genetic and plastic response are unlikely to shift CTmax enough to keep pace with predicted climate change.

The limited potential for phenotypic plasticity to increase CTmax could be explained by the trade-off hypothesis, which predicts that a trade-off between high basal thermal tolerance and plasticity may constrain the evolution of thermal plasticity (Stillman 2003; Kellett, Hoffmann & McKechnie 2005; Calosi, Bilton & Spicer 2008; Somero 2010). Empirical support for the trade-off hypothesis is mixed (Stillman 2003; Kellett, Hoffmann & McKechnie 2005; Calosi, Bilton & Spicer 2008; Mitchell, Sgrò & Hoffmann 2011; Magozzi & Calosi 2015). This may in part be attributed to the fact that past studies have directly compared basal and hardened heat tolerance (but see Stillman 2003), which may produce spurious support for trade-offs because the two traits are not independent (Kelly & Price 2005). Here we take a different approach and relate hardening capacity to developmental temperate. Doing so reveals a significant negative association between hardening capacity and developmental temperature, in contrast to a positive association between basal CTmax and developmental temperature. Thus, flies with the highest levels of basal tolerance at warmer temperatures also have the lowest hardening capacity, consistent with the trade-off hypothesis. These associations suggest that although hardening may increase CTmax at lower temperatures, thermal plasticity in response to warmer developmental temperatures is unable to shift CTmax much beyond basal levels. Congruent with these results, experimental evolution experiments in *D. melanogaster* showed that lines with higher evolved basal heat resistance showed the lowest hardening capacity (Cavicchi *et al*. 1995; Bettencourt, Feder & Cavicchi 1999). These results suggest that the most heat-tolerant taxa may be just as vulnerable to warming as less tolerant taxa because they are already close to their upper thermal limit, and their scope for plastic responses are more limited.

In contrast to theory that predicts a positive relationship between seasonal (latitudinal) variability and plasticity (Janzen 1967), we found no difference in hardening response between our temperate and tropical populations. Sgrò *et al*. (2010) observed a weak negative association between hardening response and latitude (a proxy for seasonal variability) in *D. melanogaster* populations also sampled from the east coast of Australia. These differences may reflect the fact that fewer populations were investigated in the current study, or the fact that we examined upper thermal limits using a dynamic ramping assay, rather than a static assay (Sgrò *et al*. 2010). We did however find a greater acclimation capacity for Melbourne under fluctuating, but not constant, developmental temperatures, as predicted by the seasonal variability hypothesis. Previous studies have found that exposure to constant vs. fluctuating temperature can affect thermal tolerance and plastic responses (Bozinovic *et al*. 2011; Fischer *et al*. 2011; Foray, Desouhant & Gibert 2014; Colinet *et al*. 2015), although the mechanisms underlying these differences remain unknown. Nonetheless, we found little evidence to suggest that organisms from tropical environments will be more at risk from climate change because of low levels of plasticity, consistent with previous work (Brattstrom 1970; Hoffmann & Watson 1993; Calosi *et al*. 2010; Sgrò *et al*. 2010; Mitchell, Sgrò & Hoffmann 2011; Overgaard *et al*. 2011; van Heerwaarden *et al*. 2014; but see Tsuji 1988; Ghalambor *et al*. 2006; Seebacher, White & Franklin 2015). Whether this pattern extends to populations of other *Drosophila* species endemic to Australia is not clear, although similar plastic responses in heat tolerance have been observed between temperate and tropical species of *Drosophila* from the East coast of Australia in studies which included endemic and introduced species (Mitchell, Sgrò & Hoffmann 2011; Overgaard *et al*. 2011).

The goal of the current study was to also investigate the maximum potential for plasticity to increase CTmax in

response to both short-term hardening and developmental acclimation treatments. In particular, we wanted to assess the maximum plastic response possible under conditions that reflect thermal variation in nature, so we examined a large number of acclimation developmental temperatures (six constant and four fluctuating) and different hardening exposure times. Unfortunately, it was not logistically feasible to simultaneously examine different hardening temperatures, which could influence hardening responses at different acclimation temperatures (Lerman & Feder 2001). Nonetheless, increasing the hardening exposure time past one hour did not increase the hardening response (Fig. S1), suggesting that we did capture the maximum hardening response possible in the populations examined. In addition, the current study only investigated plastic effects on CTmax at the adult stage, despite the fact that different life stages of an organism may inhabit different habitats, experience different microclimates and have different thermal sensitivities and plastic responses (Coyne, Bundgaard & Prout 1983; Kingsolver *et al*. 2011; Briscoe *et al*. 2012; van Heerwaarden *et al*. 2014; Pincebourde & Casas 2015). Whether plasticity for upper thermal limits in other developmental stages is similarly constrained is not clear. Furthermore, the extent to which plasticity itself may evolve (Scheiner & Lyman 1991), or whether cross-generational plasticity (Crill, Huey & Gilchrist 1996; Hoffmann, Chown & Clusella-Trullas 2013) may further buffer species from increasing temperatures, is unknown (Sgrò, Terblanche & Hoffmann 2016).

Despite detecting plasticity in upper thermal limits, hardening and acclimation responses were small, improving CTmax by a maximum of 1 °C across both populations. If we consider our results together with those from Hangartner & Hoffmann (2015) who showed that *D. melanogaster* has the potential to increase CTmax by 0·5 °C via evolutionary shifts, this species might have the capacity to increase upper thermal tolerances by about 1·0–1·5 °C. These results suggest that *D. melanogaster* could keep up with climate change for several decades, but that even this highly tolerant species may be limited in its response to a 2–4 °C increase. Overall, these results suggest that overheating risk in *D. melanogaster* under climate change will be minimally reduced by plasticity in the longer term. Furthermore, evidence of a trade-off between basal heat resistance and hardening capacity at warmer temperatures indicates that *Drosophila* species that are the most heat-tolerant may be at a greater risk from warming because they are already close to their upper thermal limit (Kellermann *et al*. 2012) and their scope for evolutionary (Hangartner & Hoffmann 2015) and plastic responses (this study) is limited.

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Data accessibility

Data deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.t9t28 (van Heerwaarden, Kellermann & Sgrò 2016).

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Supporting Information

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

Table S1. Temperature regime for fluctuating temperature treatment.

Table S2. Analysis of variance examining differences in absolute hardening capacity between average developmental temperature, treatment (fluctuating and constant) and population.

Table S3. Analysis of variance examining differences in relative hardening capacity between average developmental temperature, treatment (fluctuating and constant) and population.

Fig. S1. CTmax for Melbourne (top) and Innisfail (bottom) flies developing under different fluctuating developmental acclimation temperatures and heat hardened at 37 °C for different durations of 30 min, 45 min, 1 h and 1 h 30 min.

