JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.13303

Evidence for lower plasticity in CT_{MAX} at warmer developmental temperatures

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Keywords:

climate change;
climatic variability;
CT_{MAX};
phenotypic plasticity.

Abstract

Understanding the capacity for different species to reduce their susceptibility to climate change via phenotypic plasticity is essential for accurately predicting species extinction risk. The climatic variability hypothesis suggests that spatial and temporal variation in climatic variables should select for more plastic phenotypes. However, empirical support for this hypothesis is limited. Here, we examine the capacity for ten *Drosophila* species to increase their critical thermal maxima (CT_{MAX}) through developmental acclimation and/or adult heat hardening. Using four fluctuating developmental temperature regimes, ranging from 13 to 33 °C, we find that most species can increase their CT_{MAX} via developmental acclimation and adult hardening, but found no relationship between climatic variables and absolute measures of plasticity. However, when plasticity was dissected across developmental temperatures, a positive association between plasticity and one measure of climatic variability (temperature seasonality) was found when development took place between 26 and 28 °C, whereas a negative relationship was found when development took place between 20 and 23 °C. In addition, a decline in CT_{MAX} and egg-to-adult viability, a proxy for fitness, was observed in tropical species at the warmer developmental temperatures (26–28 °C); this suggests that tropical species may be at even greater risk from climate change than currently predicted. The combined effects of developmental acclimation and adult hardening on CT_{MAX} were small, contributing to a <0.60 °C shift in CT_{MAX}. Although small shifts in CT_{MAX} may increase population persistence in the shorter term, the degree to which they can contribute to meaningful responses in the long term is unclear.

Introduction

Upper thermal limits are a critical trait shaping the capacity of species to survive under climate change (Overgaard *et al.*, 2014). Interspecific studies on ectotherms, however, reveal little variation in upper thermal limits (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008; Kellermann *et al.*, 2012b). An absence of variation at the species level could be driven by a lack of selection acting on upper thermal limits resulting from behavioural thermal regulation (i.e. Bogert effect) (Huey *et al.*, 2012; Kellermann *et al.*, 2012b). Behavioural thermoregulation may ameliorate the effects of climate change in some organisms, but the extent to which small ectotherms, such as *Drosophila*, can utilize behavioural thermoregulation is less certain (Sunday *et al.*, 2014), and will depend on climate change effects on habitat heterogeneity (Caillon *et al.*, 2014). Fundamental constraints caused by an absence of genetic variation upon which selection can act could also drive the lack of variation in upper thermal limits (Chown *et al.*, 2010; Hoffmann, 2010). In *D. melanogaster*, plateaued selection responses (Gilchrist & Huey, 1999; Hangartner & Hoffmann, 2016) and an absence of genetic variation in some family studies (Mitchell & Hoffmann, 2010) lend support to the fundamental constraints hypothesis.

A lack of variation in upper thermal limits (basal CT_{MAX}) has been shown (Addo-Bediako *et al.*, 2000;

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Kellermann *et al.*, 2012b), but whether species vary in their capacity to increase their CT_{MAX} via phenotypic plasticity is less certain. The capacity for species to respond to climate change via plasticity may be critical to a species persistence (Sgro *et al.*, 2016; Chevin & Hoffmann, 2017), yet estimates of climate change risk fail to account for plasticity (Tewksbury *et al.*, 2010; Kellermann *et al.*, 2012b; Sunday *et al.*, 2014). For instance, recent studies suggest that both tropical and subtropical species are at the greater risk of extinction under climate change (Deutsch *et al.*, 2008; Clusella-Trullas *et al.*, 2011; Kellermann *et al.*, 2012b), yet they ignore the extent to which this risk might be ameliorated by plasticity. However, the capacity for species to respond via plasticity may not be randomly distributed across habitat types. The climatic variability or latitudinal hypothesis predicts that selection for plasticity should be strongest in temporally and spatially varying environments, meaning that temperate/subtropical species should evolve high plasticity to counter large seasonal/daily fluctuations, whereas tropical should evolve low plasticity (Gabriel *et al.*, 2005; Gunderson & Stillman, 2015). Theoretical models support this hypothesis, predicting that plasticity should evolve when there is spatial and temporal environmental heterogeneity, when the environmental cues are predictable and the cost to plasticity is low (Janzen, 1967; Gabriel & Lynch, 1992; Gavrilets & Scheiner, 1993; Gabriel *et al.*, 2005; Ghalambor *et al.*, 2006). However, empirical support for the climatic variability hypothesis is equivocal (Stillman, 2003; Calosi *et al.*, 2008; Mitchell *et al.*, 2011; Overgaard *et al.*, 2011; Gunderson & Stillman, 2015; Seebacher *et al.*, 2015; Pereira *et al.*, 2017) and whether tropical species have a reduced capacity to mount plastic responses that shift their upper thermal limits compared to their more temperate/subtropical counterparts remains to be determined.

Treatments that induce plastic responses are often divided into short- and long-term exposures to temperature encompassing weeks, days or hours. Longer treatments (days, weeks) tend to examine the effects of less stressful temperatures on traits and often include developmental (developmental acclimation) and adult (adult acclimation) exposures; such acclimation treatments may induce irreversible and reversible plastic responses, respectively (Hoffmann *et al.*, 2003; Piersma & Drent, 2003). Shorter exposures (minutes–hours) at more stressful temperatures are often referred to as hardening. Hardening responses in heat resistance have been extensively studied in *Drosophila* and are linked to the transient upregulation of heat-shock proteins (Telonis-Scott *et al.*, 2014; Willot *et al.*, 2017). In ectothermic species, temperature acclimation (both developmental and adult) and adult hardening treatments increase heat resistance with increasing temperature (Chown, 2001; Hoffmann *et al.*, 2003). However, different forms of plasticity (developmental/adult acclimation) can contribute unequally to shifts in CT_{MAX} (Schaefer & Ryan, 2006; Watkins & Vraspir, 2006; Slotsbo *et al.*, 2016; Kellermann *et al.*, 2017). For example, in *D. melanogaster* developmental acclimation contributes to larger shifts in CT_{MAX} than adult acclimation (van Heerwaarden *et al.*, 2016).

Intra- and interspecific studies examining the capacity for *Drosophila* species to increase their heat resistance via developmental and adult acclimation and/or hardening responses have produced mixed results (Hoffmann *et al.*, 2005; Kellett *et al.*, 2005; Sgro *et al.*, 2010; Mitchell *et al.*, 2011; Overgaard *et al.*, 2011; van Heerwaarden *et al.*, 2016; Schou *et al.*, 2017). At the intraspecific level, a weak negative association between plasticity (measured as an adult hardening responses) and latitude was detected in *D. melanogaster*, whereby tropical populations were more plastic than their subtropical/temperate counterparts (Sgro *et al.*, 2010), which is inconsistent with the climatic variability hypothesis. At the interspecific level, significant adult hardening responses in heat knockdown have been shown in a number of *Drosophila* species (Kellett *et al.*, 2005; Mitchell *et al.*, 2011); although plasticity was only weakly associated with the environment (southern latitude used as a proxy for environment) in one study (Mitchell *et al.*, 2011), such that tropical species tended to be less plastic than their widespread counterparts. Significant developmental acclimation effects have also been detected at the interspecific level in *Drosophila* for CT_{MAX}, but plasticity was unrelated to the species' environment or latitude of origin (Overgaard *et al.*, 2011; Schou *et al.*, 2017). These results suggest that *Drosophila* species, in general, have varying capacities to increase their upper thermal limits via plasticity and that this variation is unlikely to be predictably shaped by environmental selection (for further discussion, see Sorensen *et al.*, 2016a). Results from *Drosophila* are consistent with results for the European diving beetle (Calosi *et al.*, 2010) and ectotherms more broadly (Gunderson & Stillman, 2015), although (Seebacher *et al.*, 2015) found greater metabolic rate compensation (linked to thermal tolerance) and thus greater plasticity in low, compared to high, latitude ectothermic species.

The inconsistencies in the above studies could be driven by a number of factors. Primarily, the number of treatments used to assess plasticity is often fewer than four (Mitchell *et al.*, 2011; Overgaard *et al.*, 2011; Gunderson & Stillman, 2015; Seebacher *et al.*, 2015), meaning that plastic responses are often assumed to be linear, even though plastic responses often take on a quadratic shape (Murren *et al.*, 2015; but see Schou *et al.*, 2017). Failure to capture the range of the plastic response may result in vastly different conclusions being made, particularly if plasticity increases/decreases beyond the thermal window examined. In addition, assessments of plasticity are often performed using constant temperature regimes (Kellett *et al.*, 2005; Mitchell

et al., 2011; Schou *et al.*, 2017), which may impact the capacity of species to respond via phenotypic plastically (the climatic variability hypothesis predicts thermally heterogeneous environments select for plasticity). Results from studies using constant temperature regimes often fail to mirror those that use fluctuating regimes (Colinet *et al.*, 2015; Kellermann *et al.*, 2015); Salachan & Sorensen, 2017 and Sorensen *et al.*, 2016b found plasticity of CT_{MAX}, in *D. melanogaster* was higher when flies were developed and adult acclimated in fluctuating environments, whereas another study found lower plasticity (van Heerwaarden *et al.*, 2016). Importantly, studies tend to examine one form of plasticity, failing to account for the combined effects of both short- and long-term plastic responses, despite all forms of plasticity contributing to climate change responses (Colinet *et al.*, 2015; Sgro *et al.*, 2016).

In this study, we comprehensively assessed, for the first time, the extent to which both developmental and adult hardening will contribute to shifts in CT_{MAX} in ten *Drosophila* species that vary in their distributions and sensitivity to thermal stress. Using four fluctuating temperature regimes, likely to be experienced in the field (temperature range 15–33 °C), we focused on the capacity for species to shift their CT_{MAX} via both developmental acclimation and adult hardening. We examined the combined effects of developmental acclimation and hardening capacity because we believe both of these forms of plasticity will be important for responding to future environmental extremes (Chown, 2001). We also examine the fitness consequences of the fluctuating thermal regimes used to assess plasticity, as responses to climate change will also depend on the capacity of species to maintain fitness with increasing temperature. Finally, to determine whether plasticity varies in a predictable manner with climate, and test the climatic variability hypothesis, we relate plasticity in CT_{MAX}, to climatic variables while accounting for phylogenetic effects.

Materials and methods

Drosophila species maintenance, collection sites and experimental set-up

Species varying in their distribution were collected from the field between 2013 and 2015, with experiments completed in 2015–2016 (Table S1). Flies were collected via sweep netting over banana bates, with females set up as iso-female lines and returned to the laboratory. Mass-bred populations of each species were initiated at the F_2 generation by pooling ten virgin females and males from each of 20 iso-female lines. Species were maintained at a population size of approximately ~750, on a yeast potato media at 25 °C with a 12 : 12 h light cycle and maintained for a minimum of two generations as a mass-bred population prior to commencement of experiments.

Developmental acclimation and hardening treatments

Developmental acclimation

To examine the effects of developmental temperatures on adult heat resistance (CT_{MAX}), adult female flies were developed from egg to adults under four fluctuating thermal regimes with different average daily means and temperatures fluctuating 5 °C either side of the mean: 20 °C (15–25 °C), 23 °C (18–28 °C), 26 °C (21–31 °C) and 28 °C (23–33 °C) (Table S2), cabinets were accurate to 0.5 °C of the set temperature. These thermal regimes represent temperatures experienced in the field during the warmer (summer and spring) months in Australia (www.bom.gov.au) and do not capture extreme temperatures. Prior to the experiments, density was controlled by picking 40 eggs into 20 vials per thermal regime. Because developmental time varies across the thermal regimes, egg picking was staggered so that adult flies would eclose from each of the temperature regimes on the same day. Two days post-eclosion, the sexes were separated using CO_2 anaesthesia, and females were given 2 days to recover before exposure to the adult hardening treatments (see below). Thus, flies were mated and between 5 and 6 days old when CT_{MAX} was assessed.

Adult heat hardening treatments

To assess the potential for species to mount a hardening response, flies were exposed to an acute heat stress for different exposure periods. The exposure periods examined were 15, 30 min, 1 and 1.5 h at 37 °C (Fig. S1). We examined the effects of different exposures times because the treatments which induce hardening responses across species is likely to differ; for instance, a 1 h exposure to 37 °C may induce a hardening response in a heat tolerant species, but may induce mortality in a less tolerant species. Any hardening treatment that induced mortality was excluded from further analyses. It is possible that the temperature chosen to harden the flies (37 °C) may influence our capacity to detect significant hardening responses. 37 °C was chosen as this temperature induces a significant hardening response in *D. melanogaster* (Telonis-Scott *et al.*, 2014), although 34 and 35 °C did not induce a hardening response in the more thermally sensitive *D. birchii* and *D. sulfurigaster* (Mitchell *et al.*, 2011). Although 37 °C is higher than temperatures commonly experienced in tropical environments (frequently observed maximum temperature ~35 °C, www.bom.gov.au), this is not outside the realm of future climate change projections which predict an increase in temperature in the Australian tropics of 1–2 °C by 2030, under an intermediate emissions scenario (RCP4.5) (www.climatechangeinaustralia.gov.au). On completion of the hardening treatments, flies were allowed to recover on food for 23 h at 25 °C. Optimal

recovery time is likely to vary between 4 and 48 h (reviewed Hoffmann *et al.*, 2003), although these results are based on heat knockdown which may have a different genetic basis to CT_{MAX} (Blackburn *et al.*, 2014). Nonetheless, as very little has been performed on the capacity of *Drosophila* species to increase their CT_{MAX} following hardening, 23 h was chosen based on pilot experiments in *D. melanogaster* (B. van Heerwaarden, personal communication).

Estimating upper thermal limits using CT_{MAX}

For each species, assessments of CT_{MAX} were completed for all treatments (developmental acclimation and adult hardening) simultaneously. Individual flies were placed into 10-mL dry vials sealed and submerging in a water bath heated to 25 °C The temperature was then gradually increased at a rate of 0.1 °C per minute, and CT_{MAX} was scored as the time and temperature at which flies went into a heat coma (i.e. no movement) (Blackburn *et al.*, 2014; Kellermann *et al.*, 2017). CT_{MAX} was assessed across two-four runs depending on the number of hardening treatments, with two-three different scorers/observers in a randomized block design.

Assessing a component of fitness under different thermal regimes: egg-to-adult viability

To link any plastic responses arising from the developmental acclimation treatments to one component of fitness, egg-to-adult viability was assessed for each species at each developmental temperature. Fifty to one hundred females were placed onto 3 laying pots containing standard fly food (see above) spread with live yeast to stimulate oviposition. For each developmental temperature and each species, twenty eggs were placed into each of 10 vials. The number of flies eclosing from each vial was scored.

Climatic variables

To examine the relationship between climatic variables, CT_{MAX} and plastic responses, we obtained distributional information from the taxodros website (http://www.taxodros.uzh.ch/) for each species. Environmental information was extracted from the worldclim dataset with environmental variables averaged across the entire distribution of a species (Hijmans *et al.*, 2005). Seven environmental variables were chosen that are thought to be important drivers of thermal tolerance and thermal plasticity in *Drosophila* species (Kellermann *et al.*, 2012a, b): (1) annual mean temperature (AMT), (2) maximum temperature of the warmest month T_{MAX}, (3) minimum temperature of the coldest month (T_{MIN}), (4) temperature seasonality (T_{SEASON}), (5) annual precipitation (P_{ANN}), (6) precipitation seasonality (P_{SEASON}) and (7) precipitation of the warmest quarter (P_{WARM}).

Analyses

To examine the effects of developmental acclimation and short-term adult hardening responses on CT_{MAX}, we first determined the treatment which induced the greatest and most consistent plastic response for each species (Table S3). Using only this treatment, we then performed, for each species, an ANOVA examining the effects of developmental acclimation, adult hardening and their interaction on CT_{MAX}. Developmental acclimation and adult hardening were deemed fixed effects, with developmental acclimation temperature treated as a continuous variable. Effects of run and scorer were treated as random variables. All analyses were performed in R (R Core Team, 2014).

Relationship between CT_{MAX}, thermal plasticity and climatic variables

Acclimation capacity

To quantify the capacity for species to respond plastically to increasing temperatures, and to relate plastic capacity back to climatic variables (and test the climatic variability hypothesis), we calculated a number of commonly used metrics (Gunderson & Stillman, 2015; van Heerwaarden *et al.*, 2016). Initially, we examined the capacity for basal flies to increase their CT_{MAX} via developmental acclimation, calculated as the maximum acclimation capacity (AC), across the minimum and maximum developmental temperatures: CT_{MAX} 28 °C − CT_{MAX} 20 °C (it is worth noting that the maximum AC is a measure of maximum plastic response in this study and may not reflect the maximum AC of the examined species in nature). To examine how AC shifts with increasing developmental temperatures, AC was also calculated for each stepwise temperature, that is CT_{MAX} 23 °C − CT_{MAX} 20 °C. To examine the combined contribution of developmental acclimation and adult hardening to CT_{MAX}, we calculated maximum hardened acclimation capacity (HAC) by examining the difference in hardened CT_{MAX} between the minimum and maximum developmental temperature (HAC: hardened CT_{MAX} 28 °C − hardened CT_{MAX} 20 °C).

To test for significant differences between the stepwise temperatures for AC, due to the high correlation between AC and HAC we present the results of AC only, we performed a one-way ANOVA for each species with temperatures deemed a fixed effect. We performed a Dunnett *post hoc* analysis comparing the stepwise acclimation treatments (i.e. 20–23 °C) to the maximum acclimation response (20–28 °C). In addition, we divided species into tropical and subtropical groups (see below for definition) and performed an ANOVA and Dunnetts *post hoc* analysis to determine whether stepwise acclimation (20–23 °C) treatments were significantly different from maximum AC (20–28 °C) within these groups, with acclimation groups defined as fixed effects.

Hardening capacity

We also examined the capacity for species to shift their CT_{MAX} via adult hardening alone. Hardening capacity (HC) was calculated within each developmental temperature as hardened CT_{MAX} − basal CT_{MAX} (van Heerwaarden *et al.*, 2016). We also considered relative hardening capacity, which standardizes HC by basal CT_{MAX}: hardened CT_{MAX} − basal CT_{MAX}/basal CT_{MAX} (Kellett *et al.*, 2005). As estimates of HC and RHC were qualitatively the same, we show the results for HC only.

Plasticity and the climatic variability hypothesis

Initially, we examined the relationship between CT_{MAX}, plasticity (AC, HC and HAC) and climate using a multiple regression approach in R. Firstly, we looked for an autocorrelation across the seven climatic variables of interest, using the variance inflation factor (VIF). A climate variable with a VIF > 5 suggests a high level of autocorrelation and were removed from the analysis (Rogerson, 2001). High autocorrelation between climate variables meant models was often reduced to two variables. Following the removal of highly autocorrelated climatic variables, we then examined the relationship between climate, CT_{MAX} and plasticity using a phylogenetic generalized least squares (pgls) approach using the caper program in R (Orme *et al.*, 2013). The phylogeny was modified from (Kellermann *et al.*, 2012a) (Fig. S2). The pgls method estimates the variance covariance matrix between traits and phylogeny; estimated through the λ parameter, $\lambda = 0$ suggests no relationship between traits and phylogeny, although $\lambda = 1$ suggests a strong relationship and traits are evolving similar to a Brownian motion model of evolution. However, having fewer than 20 species will greatly reduce the power to detect a relationship between traits and phylogeny, which is reflected in the large confidence intervals around estimates of phylogenetic signal (λ) (Blomberg *et al.*, 2003). Model fit was assessed using Akaike Information Criterion (AIC), where models had an AIC > 2.5 the simplest model with fewer variables was chosen.

Tropical vs. subtropical species

To further examine patterns in plasticity that may be linked to climatic variables, we divided species into two groups: tropical and subtropical. Here we focused our analysis on AC because plastic responses were small for HC and therefore unlikely to be the most important response to climate change, and because HAC and AC were highly autocorrelated, producing qualitatively the same result. Based on the climate data, we defined tropical species as those occupying environments between 0 and 23° latitude, annual precipitation >1500 mm/year and annual mean temperature >18 °C; temperate species as those occupying environments above 40° latitude and the remaining species were deemed subtropical (Wit *et al.*, 2015). Among the ten species, only one species was deemed a temperate species and was thus excluded from the tropical/subtropical comparisons. Of the nine, remaining species four were deemed tropical and five subtropical. To determine whether AC differed across the stepwise temperatures for the tropical and subtropical species, we performed a generalized linear model, with species and stepwise temperature deemed fixed effects. We analysed differences between the stepwise temperatures, within species climate groups, using a Bonferonni *post hoc* test. To determine how AC differed across the stepwise temperatures for each species, we performed a generalized linear model, with stepwise temperature deemed a fixed effect, followed by a Bonferonni *post hoc* test on the stepwise temperatures.

Acclimation response ratio

To further quantify the plastic response to the combined effects of development acclimation and adult hardening, we examined the acclimation response ratio (ARR, developmental acclimation only) and the hardened acclimation response ratio (HARR, developmental acclimation and adult hardened) (Gunderson & Stillman, 2015; van Heerwaarden *et al.*, 2016). This measure takes into account the degree of temperature change across the developmental acclimation treatments and is calculated as: AC/°C change between the two temperatures used to calculate AC (i.e. ARR = CT_{MAX} 23 °C − CT_{MAX} 20 °C/3 °C, HARR = hardened CT_{MAX} 23 °C − CT_{MAX} 20 °C/3 °C). ARR/HARR was calculated for each stepwise temperature change (i.e. 20–23 °C, 23–26 °C and 26–28 °C) and then averaged across these treatments to give a single value of ARR/HARR. In addition to providing a method to obtain standardized estimates of acclimation responses across temperature regimes, ARR can also be used to assess overheating risk of species (Gunderson & Stillman, 2015; van Heerwaarden *et al.*, 2016). An ARR/HARR of 1 indicates a plastic increase of 1 °C in CT_{MAX} for a 1 °C change in developmental temperature, implying that CT_{MAX} is able to perfectly track the environment. An ARR/HARR of 0 indicates that no plastic shift in CT_{MAX} with increasing developmental temperatures is possible. CT_{MAX} overheating risk was calculated as (1 − mean ARR/HARR) × change in environmental temperature (Gunderson & Stillman, 2015).

Results

Developmental acclimation and adult hardening

We examined the capacity for ten *Drosophila* species, varying in their distribution and sensitivity to heat stress, to increase their heat resistance (CT_{MAX}) via developmental acclimation and short-term adult

Table 1 Maximum acclimation capacity (AC), adult hardening capacity (HC) and acclimation and hardening capacity combined (HAC) across the developmental temperatures and the acclimation/hardening response ratio (ARR/HARR) for each of the ten species. Plasticity responses are divided into basal: estimates of CT_{MAX} on flies not exposed to hardened treatments and hardened: estimates of CT_{MAX} on flies exposed to hardening treatments.

	AC (°C) (basal)		AC (°C) (hardened)		
	AC	ARR	HC	HAC	HARR
Drosophila ananassae	0.443	0.047	0.044	0.514	0.070
D. birchii	−0.178	−0.024	0.052	0.100	0.010
D. bunnanda	0.407	0.045	0.097	0.389	0.077
D. buzzatti	0.253	0.036	0.106	0.089	0.052
D. hydei	0.327	0.046	0.064	0.404	0.045
D. immigrans	−0.330	−0.076	0.086	−0.428	−0.034
D. melanogaster	0.422	0.061	0.182	0.223	0.105
D. serrata	0.287	0.032	0.035	0.296	0.043
D. simulans	0.433	0.046	0.073	0.300	0.044
D. sulfurigaster	0.195	0.018	−0.001	0.209	0.018

hardening. Increasing developmental temperatures significantly increased heat resistance in eight of the ten *Drosophila* species (Table S3). Run and scorer had significant effects on estimates of CT_{MAX} (Table S4). Basal CT_{MAX} varied across the species (36.16–41.60 °C), with *D. immigrans* and *D. buzzatti* the least and most resistant species, respectively. The observed plastic increase in CT_{MAX} following development acclimation (AC = CT_{MAX} 28 °C − CT_{MAX} 20 °C) was small, ranging from −0.08 to 0.44 °C (Table 1). For two species, *D. immigrans* and *D. birchii*, increasing developmental acclimation temperatures significantly decreased their CT_{MAX} (Table S3).

Similar to developmental acclimation, a short nonlethal heat stress (adult hardening) significantly increased the CT_{MAX} in six of the ten *Drosophila* species (Tables 1 and S3, Fig. 1a). The effects of adult hardening were smaller than for developmental acclimation: −0.001–0.18 °C increase in CT_{MAX} (Table 1, Fig. 1a). Longer hardening treatments tended to reduce CT_{MAX}, suggesting deleterious consequences of longer heat exposures (Table S3). A significant interaction was detected between developmental acclimation and adult hardening for *D. birchii* and *D. buzzatti*. For *D. birchii*, cooler developmental temperatures (20–23 °C) decreased adult hardening capacity, whereas warmer developmental temperatures increased adult hardening capacity (Table S4). In *D. buzzatti*, increasing developmental temperatures decreased adult hardening capacity. No relationship was found between hardening capacity and developmental temperature. Of the species examined, *D. birchii* was the only species that did not significantly increase CT_{MAX} through either developmental acclimation or adult hardening (Table 1, Fig. 1a).

Excluding the two species that showed negative effects of developmental acclimation and adult hardening on CT_{MAX} (*D. birchii* and *D. immigrans*), the maximum capacity to shift CT_{MAX} through developmental acclimation and adult hardening across all species was between 0.09 and 0.51 °C (Table 1, Fig. 1a). When we compared the capacity for species to increase their CT_{MAX} to track environmental change via plasticity (i.e. ARR/HARR), we found that species had little capacity to respond plastically to increasing heat stress via developmental acclimation and adult heat hardening (Fig. S3).

Further dissection of the plastic response across all of the developmental acclimation temperatures showed that CT_{MAX} overall increased between the development temperatures 20–28 °C in eight of ten species (Fig. 2); focussing just on the warmer developmental temperatures of 26–28 °C revealed that only five of the 10 species showed a positive increase in CT_{MAX} (Fig. 2, Table S5). When we compared maximum AC (20–28 °C) to AC across the stepwise acclimation temperatures (20–23 °C, etc.), for subtropical species maximum AC was significantly higher for the stepwise AC calculated between the lower temperatures (Fig. 3, Table S6). Similarly for tropical species, maximum AC was significantly higher for all but the 20–23 °C treatment (Fig. 3), whereas AC in the 26–28 °C was negative.

The effect of developmental acclimation on egg-to-adult viability

Developmental temperature had a significant effect on egg-to-adult viability in seven of the ten species (Fig 1b, Table S7). Optimum developmental temperature for egg-to-adult viability varied considerably across the species. Overall, the highest egg-to-adult viability was found in the 23 °C developmental acclimation treatment followed closely by 20 °C, although egg-to-adult viability was lowest at the 28 °C development acclimation treatment. Subtropical species tended to have higher viability in the 28 °C treatment ($\bar{x} = 0.68 \pm 0.09$) (excluding the cold adapted species *D. immigrans*) in comparison with the tropical species ($\bar{x} = 0.44 \pm 0.12$), with egg-to-adult viability decreasing at 28 °C, in comparison with the 23 °C treatment, by 3% in the subtropical and 37% in tropical species. These results suggest greater deleterious fitness consequences for tropical species developing at warmer temperatures. The least resistant species was the temperate species *D. immigrans* with egg-to-adult viability close to 0 in the 28 °C treatment. Of the three species that did not show a significant effect of temperature on egg-to-adult viability, egg-to-adult viability for two species (*D. ananassae* and *D. simulans*) remained consistently high across the four developmental temperatures, although variation in egg-to-

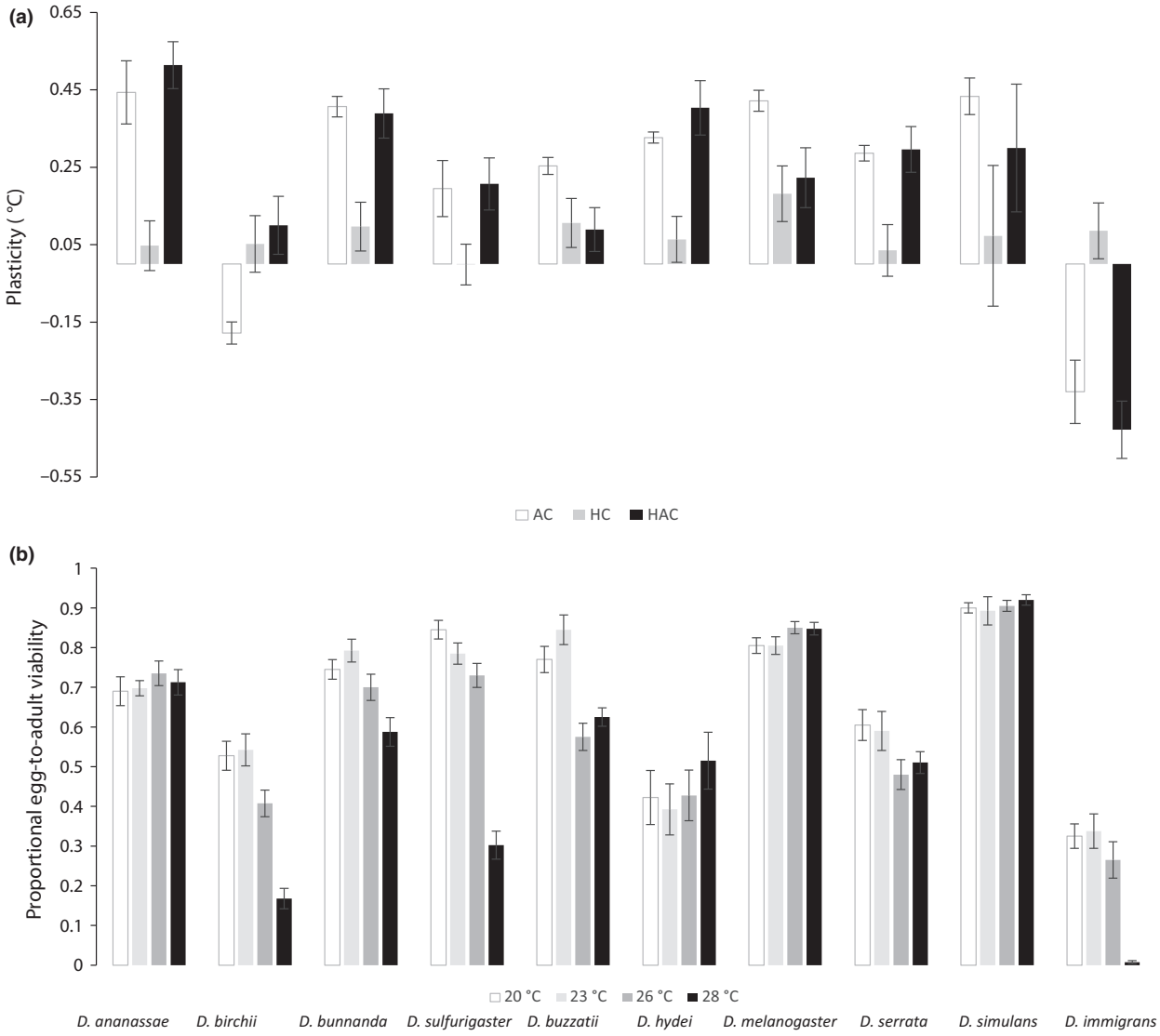

Fig. 1 (a) Maximum acclimation capacity (AC), adult hardening capacity (HC), acclimation and hardening capacity combined (HAC) and (b) egg-to-adult viability across the developmental temperatures, for all ten species.

adult viability for *D. hydei* followed no consistent pattern with temperature.

Relationship between CT_{MAX}, thermal plasticity and climatic variables

Annual precipitation (P_{ANN}) and maximum temperature (T_{MAX}) explained a significant proportion of the variation in basal CT_{MAX} in all developmental temperatures (R^2 = 0.544–0.626, Table 2), such that basal CT_{MAX} increased with increasing T_{MAX} and decreasing P_{ANN}. None of the climatic variables, however, were related to adult hardening response (HC), developmental acclimation (AC and ARR) or the combined estimates of plasticity (HAC, max AC and HARR) (Table 2). On closer examination, we did however find a significant positive relationship between T_{SEASON} and AC calculated between treatments 26 and 28 °C (R^2 = 0.502, slope = 0.00004 ± 0.00001, P = 0.020, λ = 0), such that AC increased with increasing T_{SEASON} (Fig. 4b). In contrast, we found a significant negative relationship between T_{SEASON} and AC calculated between treatments 20 and 23 °C (R^2 = 0.613, slope = −0.0005 ± 0.00001, P = 0.005, λ = 0) (Fig. 3a), such that AC decreased with increasing T_{SEASON} (Fig. 3c). This pattern was predominately driven by higher AC in the tropical species at the cooler temperatures and lower AC in the tropical species at hotter temperatures, and a tendency for subtropical species to do the opposite (Fig. 3). We found no relationship between CT_{MAX},

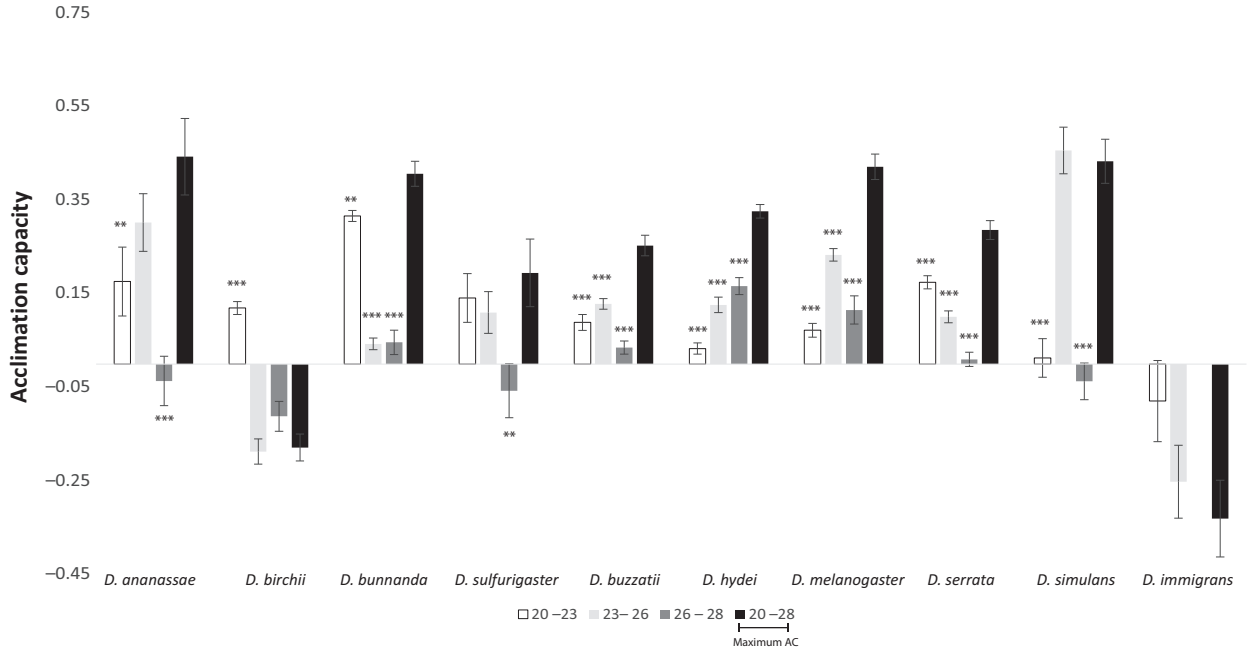

Fig. 2 Acclimation response (±1 standard error) calculated between each developmental acclimation regime, that is CT_{MAX} 23 °C–CT_{MAX} 20 °C, for each species. $^{**}P < 0.01$ and $^{***}P < 0.001$ significance of stepwise treatments to maximum acclimation response (within this study) was compared using a Dunnetts *post hoc* analysis.

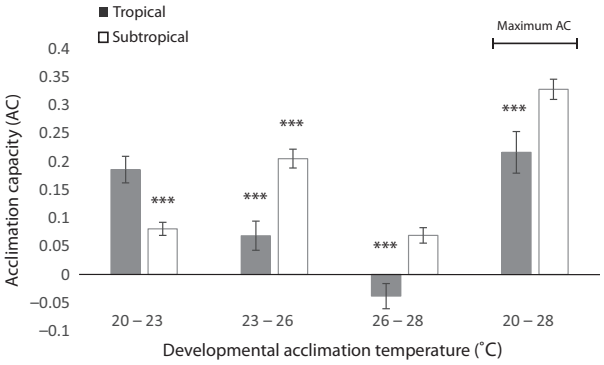

Fig. 3 Acclimation response (AC) calculated between each developmental acclimation regime i.e. CT_{MAX} 23 °C–CT_{MAX} 20 °C, for tropical and subtropical species. $^{***}P < 0.001$ significance of stepwise treatments to maximum acclimation response (within this study) was compared using a Dunnetts *post hoc* analysis.

estimates of plasticity and phylogeny as evidenced by estimates of $\lambda = 0$. Viability at all developmental temperature was unrelated to climatic variables.

Discussion

Current assessments of species climate change risk suggest that subtropical and tropical species are already experiencing temperatures beyond their current thermal limits (CT_{MAX}) (Deutsch *et al.*, 2008; Tewksbury *et al.*, 2010; Kellermann *et al.*, 2012b). However, these studies fail to account for the role of phenotypic plasticity in buffering species from thermal extremes. If species from more variable environments have greater plasticity (climatic variability hypothesis), then subtropical/temperate species may have a greater capacity to increase their CT_{MAX} via plasticity compared to their tropical counterparts (Janzen, 1967; Gabriel & Lynch, 1992; Ghalambor *et al.*, 2006), and be less vulnerable to increasing thermal stress. In the current study, using fluctuating thermal regimes, we examine the capacity for ten *Drosophila* species to increase their CT_{MAX} via both developmental acclimation and adult hardening. We also determine the role of climatic variables in shaping these two measures of plasticity, and test the climatic variability hypothesis for the evolution of plasticity. Finally, we dissect the plastic responses across developmental temperatures, revealing a level of complexity between plasticity, climate and a component of fitness (egg-to-adult viability) that would be missed with a focus on absolute measures of plasticity.

The capacity for species to increase their CT_{MAX} via the combined effects of developmental acclimation and hardening was small (<0.60 °C). Developmental acclimation had the biggest effect on CT_{MAX} (average AC = +0.22 °C), although adult heat hardening increased CT_{MAX} by on average of only <0.06 °C. A

Table 2 Phylogenetic least squares analysis examining the relationship between CT_{MAX} and acclimation response ratio (ARR) and environmental variables. Association between the phylogeny and CT_{MAX} is estimated as λ, where a $\lambda = 0$ suggests no relationship with phylogeny and $\lambda = 1$ suggests a strong relationship. Phylogenetic signal from the residuals was also estimated for CT_{MAX} and ARR using two methods the K statistic and λ.

	PGLS			Phylogenetic signal	
	R^2	Slope	λ	K	λ
CT_{MAX} 20					
P_{ANN}	0.567	−0.004 ± 0.001**	0 (0–0.823)	1.125**	1.045***
T_{MAX}		0.077 ± 0.026*			
CT_{MAX} 23					
P_{ANN}	0.544	−0.004 ± 00001*	0 (0–0.822)	1.071**	1.011**
T_{MAX}		0.081 ± 0.027*			
CT_{MAX} 26					
P_{ANN}	0.550	−0.004 ± 0.001**	0 (0–0.848)	1.068*	1.059**
T_{MAX}		0.089 ± 0.029			
CT_{MAX} 28					
P_{ANN}	0.626	−0.003 ± 0.001*	0 (0–0.512)	0.795*	0.899*
T_{MAX}		0.045 ± 0.026			
ARR					
T_{MAX}	0.001	−0.0001 ± 0.0001	0 (0–0.982)	0.429	0
Combined dataset					
CT_{MAX} 20					
P_{ANN}	0.623	−0.002 ± 0.004***	0 (0–0.979)	1.125**	1.045***
T_{MAX}		0.344 ± 0.068***			
ARR					
T_{MAX}	0.519	0.024 ± 0.005***	0 (0–0.786	0.342	0

*$P < 0.01$, **$P < 0.001$, ***$P < 0.001$.

similarly low level of variation in developmental acclimation and hardening was also shown intraspecifically in *D. melanogaster* (van Heerwaarden *et al.*, 2016). The capacity to increase CT_{MAX} via heat hardening was low across most of the *Drosophila* species examined here, and contrasts with Kellett *et al.* (2005) who found larger hardening responses that were of a similar magnitude across a number of *Drosophila* species. The observed differences are likely driven by the different methods used in the two studies – fluctuating thermal regimes and dynamic/ramping assessments of CT_{MAX} in the current study vs. constant temperature and static/knockdown assessments of CT_{MAX} in Kellett *et al.* (2005). The mechanisms underpinning the hardening responses have been well defined in *D. melanogaster* and are linked to the upregulation of Hsp70, but changes in Hsp70 expression have not been linked to hardening in other *Drosophila* species (Krebs, 1999; Hoffmann *et al.*, 2003). These results suggest that the capacity for Hsp70 to act as regulator of heat plasticity may be species-specific (Hamdoun *et al.*, 2003; Ravaux *et al.*, 2016; Willot *et al.*, 2017).

Despite plasticity only shifting CT_{MAX} by <0.60 °C, mechanistic species distribution models have shown that even a 0.50 °C change in CT_{MAX} can contribute to meaningful reductions in projected range losses under climate change (Bush *et al.*, 2016). Thus, small plastic shifts in CT_{MAX} may nonetheless be crucial in buffering species in the short term, providing an opportunity for selection to shift CT_{MAX}. That being said, the lack of variation in plasticity across the species examined is notable. A <0.60 °C plastic change in CT_{MAX} compared to basal CT_{MAX} varying ~5 °C across the species suggests that the capacity for the species examined to shift CT_{MAX} via plasticity is low. In contrast, plastic responses in CT_{MAX} in other species are higher than those observed for *Drosophila*. For instance, in lizards heat hardening can increase CT_{MAX} by 2.6 °C, although trade-offs with basal CT_{MAX} is suggestive of an upper limit to hardening responses (Phillips *et al.*, 2016). In addition, developmental acclimation and hardening can also increase CT_{MAX} in the copepod *Tigriopus californicus*, the springtail *Orchesella cincta* and the moth *Manduca sexta* by 1.3 °C, ~1.5 °C and ~1.2 °C, respectively (Kingsolver *et al.*, 2016; Alemu *et al.*, 2017; Pereira *et al.*, 2017). It is possible that more extreme temperatures,

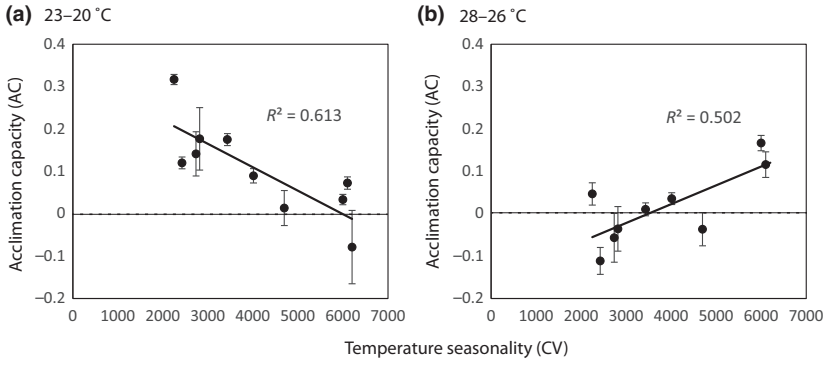

Fig. 4 The relationship between environment (T_{SEASON}) and developmental acclimation capacity between (a) 20 °C and 23 °C (basal CT_{MAX} 23 °C–basal CT_{MAX} 20 °C) and (b) 26 °C and 28 °C (basal CT_{MAX} 28 °C–basal CT_{MAX} 26 °C).

than imposed in the current experiment, may induce greater plastic responses in some species. Given, however, we observed declines in fitness in the tropical species and hardening responses were small, more extreme thermal regimes are unlikely to significantly increase the plastic response. Whether the observed plastic responses are large enough to keep pace with climate change remains to be determined, but suggests that other species have a greater plastic capacity for CT_{MAX} than *Drosophila*.

We found a strong relationship between basal CT_{MAX} and climate (T_{MAX} and P_{ANN}) across all developmental temperatures, as well as a strong association between phylogeny and CT_{MAX}. Strong phylogenetic signal in CT_{MAX} is consistent with previous work in examining ~90 species of *Drosophila* (Kellermann *et al.*, 2012b) (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008). However, we found no association between plasticity (AC, ARR, HC, HRR), phylogeny and any climatic variables when averaging the plastic response across all thermal regimes. Thermal plasticity therefore does not vary predictably with the climate, a result inconsistent with the climatic variability hypothesis. However, dissecting the plastic response across acclimation temperatures (20–23 °C, 23–26 °C and 26–28 °C) revealed a more complex interplay between climatic variables and plasticity that was not apparent when absolute (28–20 °C) and standardized (ARR) measures of plasticity were used. Specifically, a significant association was found between temperature seasonality and AC, but this association differed depending on the developmental temperature range considered (positive for 26–28 °C, negative for 20–23 °C Fig. 3). One possible explanation for this shift in the relationship between plasticity and climate with the thermal range is that species with a higher overall basal CT_{MAX} (subtropical species) may require warmer developmental temperatures to induce a plastic response in CT_{MAX} in comparison with species with lower basal CT_{MAX} (tropical species), although plastic responses are induced at lower thermal ranges in the less heat resistant tropical species. Indeed, subtropical species with on average higher CT_{MAX} tended to have a lower AC at lower developmental temperatures in comparison with tropical species and vice versa at higher developmental temperatures (Fig. 1). How a plastic response is induced and whether the induction of plasticity is linked to a species basal stress response still remains to be determined. Hsp70 is one possible candidate, but we still have limited insight into the mechanisms that underpin thermal plasticity.

Despite finding significant AC between 20 and 28 °C for subtropical and tropical species, in three of the four tropical species, CT_{MAX} declined between the warmest temperatures (26–28 °C) (Figs 2 and S1). Declines in CT_{MAX} coupled with declines in egg-to-adult viability of 37% in the warmest treatment (28 °C) highlights the compounding effects of warm temperatures on tropical species. These results, coupled with the opposing relationship between climate and plasticity dependent on the temperature range examined, suggest that standardized measures of plasticity, which average estimates of plasticity across temperatures, may not capture the real extent of plastic responses, and may hide ecologically meaningful patterns. The lack of consistent associations between climate and plasticity, shown in previous studies, could in part be driven by the thermal range examined and by averaging plasticity across thermal regimes (Stillman, 2003; Mitchell *et al.*, 2011; Overgaard *et al.*, 2011; Gunderson & Stillman, 2015).

It is worth noting that here we examined only a single population of each species and thus ignore the potential for intraspecific variation in plastic responses across the species. However, variation in plasticity in CT_{MAX} between a northern and southern population of *D. melanogaster* was no greater than the variation observed at the species level in the current study (van Heerwaarden *et al.*, 2016), and thus, we expect that intraspecific variation will be smaller than interspecific variation (Kimura, 2004). We focused on warmer developmental temperatures and included fluctuating thermal regimes that ranged from 15 to 33 °C because we were interested in the ability of species to respond plastically to increasing temperatures as projected under climate change. This means that our absolute estimates of plasticity may be an underestimate as we do not include the entire developmental range of all the species examined. Nonetheless, we believe that our results have revealed new insight into the combined effects of developmental acclimation and adult hardening on upper thermal limits in small terrestrial ectotherms. It is possible that laboratory adaptation and inbreeding may be an inherent component of the current experiment. However, lengths were taken to minimize these effects: most species (seven) were assayed within 1 year from collection from the field, limiting the potential for laboratory adaptation to drive the observed results (Table S1), and mass-bred populations were initiated with 20 iso-female lines and maintained at high population sizes (>750 individuals) to avoid inbreeding. Furthermore, recent work suggests laboratory maintenance does not have large effects on physiological patterns in *Drosophila* (Maclean *et al.*, 2018). In addition, previous studies (van Heerwaarden & Sgro, 2011; Blackburn *et al.*, 2014) show that populations maintained under these conditions and for similar timescales harbour significant levels of adaptive genetic diversity.

In conclusion, we have shown that variation in plasticity in CT_{MAX}, when estimated in fluctuating environments, was low across all species, particularly at warmer developmental temperatures, which resulted in deleterious effects on CT_{MAX} and viability in tropical species. This suggests that the capacity for plasticity to contribute to meaningful shifts in CT_{MAX} under climate change is likely to be low generally, and even lower for

tropical species. In contrast to the climatic variability hypothesis, we found no association between overall estimates of plasticity and climatic variability. However, the relationship between climate and plasticity was complex and differed depending on the extent of the developmental thermal range used to quantify plasticity, and the number of developmental temperatures examined. Thus climatic variability, *per se*, does not seem to easily predict the scope for thermal plasticity. Such a complex relationship between climatic variables and plasticity may contribute to the lack of consistent support for the climatic variability hypothesis in the literature more broadly (Stillman, 2003; Calosi *et al.*, 2008; Mitchell *et al.*, 2011; Overgaard *et al.*, 2011; Gunderson & Stillman, 2015; Seebacher *et al.*, 2015) and suggests that plasticity is unlikely to be easily predicted by climatic factors alone.

Acknowledgments

We are grateful to Belinda van Heerwaarden, Katherine Sutton, Fiona Beasley and Lindsey Heffernan for technical assistance, the Australian Research Council for financial support to VK and CMS through their Discovery and Fellowship schemes and the Science and Industry Endowment Fund for support to CMS, Monash University for financial support to VK and CMS and L'Oreal for financial support to VK through their For Women In Science scheme.

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Supporting information

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

Figure S1 Experimental design for adult hardening and developmental acclimation treatments.

Figure S2 Phylogenetic hypothesis for the ten *Drosophila* species examined.

Figure S3 The contributions of developmental acclimation and adult heat hardening to CT_{MAX} to a 1–5 °C change in temperature, expressed as overheating risk i.e. the difference between mean environmental temperature and CT_{MAX}. The thin solid line represents the overheating risk when plasticity (developmental and/or hardening) does not contribute to increases in CT_{MAX}, while the thick black line represent the predicted response when a species can perfectly compensate to changes in their environment via plasticity. The coloured dashed lines represent one of the 10 species examined.

Table S1 Collection, site, date of collection and date of experiment for each of the 10 *Drosophila* species examined.

Table S2 Thermal regimes for each cabinet.

Table S3 Analysis of variance examining the effects of adult hardening for each developmental acclimation regime for each species and Dunnets *post hoc* comparison of hardening treatments relative to the control. The average difference between treatments is shown as the mean of the controls minus mean of the hardening treatments (C-T).

Table S4 Analysis of variance testing for the effects of developmental acclimation and adult hardening for each species.

Table S5 Analysis of variance examining the effect of acclimation treatments on plastic responses for each species.

Table S6 Analysis of variance examining the effect of acclimation treatments (20–23 °C, 23–26 °C, 26–28 °C and 20–28 °C) on plastic response. Species were divided into two habitat types: tropical and subtropical based on their current distribution.

Table S7 Analysis of variance examining the effect of developmental temperatures on egg-to-adult viability in the 10 *Drosophila* species.

Data deposited at Dryad: https://doi.org/10.5061/dryad.56ck582

Received 21 March 2018; revised 27 May 2018; accepted 29 May 2018