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Multivariate analysis of adaptive capacity for upper thermal limits in *Drosophila simulans*

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Abstract

Thermal tolerance is an important factor influencing the distribution of ectotherms, but our understanding of the ability of species to evolve different thermal limits is limited. Based on univariate measures of adaptive capacity, it has recently been suggested that species may have limited evolutionary potential to extend their upper thermal limits under ramping temperature conditions that better reflect heat stress in nature. To test these findings more broadly, we used a paternal half-sibling breeding design to estimate the multivariate evolutionary potential for upper thermal limits in *Drosophila simulans*. We assessed heat tolerance using static (basal and hardened) and ramping assays. Our analyses revealed significant evolutionary potential for all three measures of heat tolerance. Additive genetic variances were significantly different from zero for all three traits. Our **G** matrix analysis revealed that all three traits would contribute to a response to selection for increased heat tolerance. Significant additive genetic covariances and additive genetic correlations between static basal and hardened heat-knockdown time, marginally nonsignificant between static basal and ramping heat-knockdown time, indicate that direct and correlated responses to selection for increased upper thermal limits are possible. Thus, combinations of all three traits will contribute to the evolution of upper thermal limits in response to selection imposed by a warming climate. Reliance on univariate estimates of evolutionary potential may not provide accurate insight into the ability of organisms to evolve upper thermal limits in nature.

Introduction

The distribution and abundance of many species, particularly ectotherms, is influenced by temperature (Cossins & Bowler, 1987). With temperatures expected to increase across the globe over coming decades (IPCC, 2007), temperature is increasingly likely to be a source of strong selective pressure for many organisms, particularly ectotherms. The close association between environmental and body temperatures means that climate change is likely to impact ectotherms' distribution and abundance (Parmesan & Yohe, 2003; Colwell *et al.*, 2008; Chown *et al.*, 2010), metabolism (Dillon *et al.*, 2010) and therefore risk of extinction (Deutsch *et al.*, 2008; Huey *et al.*, 2009; Sinervo *et al.*, 2010). Importantly, a relationship between upper thermal tolerance and maximum habitat temperature has been demonstrated for many species (Tomanek & Somero, 1999; Stillman & Somero, 2000; Stillman, 2002; Sinervo *et al.*, 2010; Somero, 2010; Duarte *et al.*, 2012), suggesting that some ectotherms already exist close to upper thermal thresholds (Stillman, 2002; Mercader & Scriber, 2008; Jones *et al.*, 2009), and are thus at greater risk of extinction from climate change.

Behavioural thermoregulation may have a limited abilty to ameliorate the effects of climate warming in ectotherms (Huey & Pascual, 2009; Huey & Tewksbury, 2009; Kearney *et al.*, 2009; Rego *et al.*, 2010), and temperature is expected to impose significant selection pressures on both ectotherms and endotherms (e.g. Huey *et al.*, 2012). Yet, whether organisms are able to modify upper thermal limits via evolutionary responses or phenotypic plasticity and mediate their extinction risk

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remains largely unknown. Recent work based on interspecific studies of *Drosophila* (Kellermann *et al.*, 2012) suggets that some organisms will be unlikely to mediate the effects of global warming via evolutionary changes. Comprehensive intraspecific assessments of adaptive capacity for upper thermal limits, which provide important insight into contemporary microevolutionary responses to global change, are more limited, and provide mixed support for such conclusions.

Compounding this issue is the fact that it has become clear that estimates of upper thermal limits can vary depending on the methodology used (Terblanche *et al.*, 2007; Mitchell & Hoffmann, 2010; Sgrò *et al.*, 2010; Rezende *et al.*, 2011; Santos *et al.*, 2011). Specifically, thermal tolerance can be assessed using either static (constant temperature) assays (Hoffmann *et al.*, 2002, 2003) or dynamic (variable temperature) assays that involve gradually heating or cooling an animal from a particular starting temperature until physiological failure, such as knockdown or loss of righting ability (Terblanche *et al.*, 2007; Mitchell & Hoffmann, 2010; Sgrò *et al.*, 2010; Overgaard *et al.*, 2012). Ramping assays are argued to be more ecologically relevant because they are thought to better reflect changes in temperature in the field and because they indicate the activity range for a population under acute conditions experienced in nature. However, the rate of change in temperature used in these assays has been shown to affect predictions about lower (Kelty & Lee, 2001; Overgaard *et al.*, 2006; Jumbam *et al.*, 2008) and upper (Mitchell & Hoffmann, 2010; Sgrò *et al.*, 2010) thermal limits. Such results have raised questions about the extent to which different measures adequately or accurately assess upper thermal limits (Rezende *et al.*, 2011; Santos *et al.*, 2011; Terblanche *et al.*, 2011; van Heerwaarden *et al.*, 2012; Overgaard *et al.*, 2012).

Importantly, inferences about an organism's ability to adapt to stressful environmental conditions via evolutionary change may also be affected by the methodology used (Chown *et al.*, 2009; Mitchell & Hoffmann, 2010; Rezende *et al.*, 2011). Numerous studies have examined genetic variation for heat tolerance in *Drosophila* (e.g. Coyne *et al.*, 1983; Jenkins & Hoffmann, 1994; Cavicchi *et al.*, 1995; McColl *et al.*, 1996; Bubliy *et al.*, 1998, 2012; Gilchrist & Huey, 1999; Krebs & Thompson, 2006; Sorensen *et al.*, 2007; Sisodia & Singh, 2010) and other taxa (e.g. (Bennett & Lenski, 1993; Neargarder *et al.*, 2003; Elderkin *et al.*, 2004; Winne & Keck, 2005; Willett, 2010; Doyle *et al.*, 2011; Kelly *et al.*, 2012). Those studies that have used multiple assay methods have shown that different measures of thermal tolerance are unrelated to each other (Hoffmann *et al.*, 1997; Sorensen *et al.*, 2001; Folk *et al.*, 2007; Sgrò *et al.*, 2010 but see Berrigan, 2000). This suggests that at least partially independent mechanisms of resistance are involved in those measures. However, none of these studies have directly addressed the question of whether static vs. the increasingly used dynamic measures provide the same information about the capacity for thermal adaptation within and between populations.

It has been suggested that dynamic assays may lower adaptive capacity for upper thermal limits (measured as the univariate narrow-sense heritability) by either increasing the environmental variance (Chown *et al.*, 2009) or by increasing the environmental variance and decreasing the additive genetic variance (Rezende *et al.*, 2011). Only one study has tested these predictions. Mitchell & Hoffmann (2010) found that compared with static measures of heat tolerance, the narrow-sense heritability and evolvability of ramping heat tolerance was significantly reduced in two populations of *D. melanogaster*. This was driven by significantly lower levels of additive genetic variance, and not inflated environmental variance, which contrasts with the predictions by Chown *et al.* (2009) and Rezende *et al.* (2011). These results imply a constrained evolutionary response to selection imposed by the gradual heating that may typically be experienced in natural populations (Hoffmann, 2010; van Heerwaarden *et al.*, 2012). If these results hold true more broadly, the capacity for evolutionary change in response to selection might be overstated if only static measures of thermotolerance are relied upon.

The static and ramping measures of heat tolerance were positively correlated across *Drosophila* species, suggesting perhaps some potential for correlated responses to selection (Mitchell & Hoffmann, 2010); however, no such association was found in an intraspecific study of *D. melanogaster* populations from along a climatic gradient (Sgrò *et al.*, 2010). However, no study has directly estimated the extent to which static vs. ramping measures of heat tolerance share a genetic basis. This is surprising, because we know that the ability of a population to respond to selection for increasing heat tolerance will be determined by the patterns of genetic variation and covariation in the traits under selection (Blows & Hoffmann, 2005). If both static and dynamic measures of thermal tolerance significantly covary with one another, then reliance on univariate measures of adaptive capacity may provide inaccurate information about the evolution of upper thermal limits under climate change.

The focus of this study was to examine the extent to which static and dynamic measures of heat tolerance reflect a shared genetic basis to upper thermal limits, and to determine whether the evolution of heat tolerance in natural populations might be constrained by low additive genetic variances or covariances between different measures of heat tolerance. Specifically, we estimated additive genetic variances and covariances for three commonly used measures of heat tolerance, static basal and hardened heat-knockdown time and ramping (dynamic) heat-knockdown time, in *Drosophila simulans*.

Previous work (van Heerwaarden *et al.*, 2012) has shown that all three measures of heat tolerance show parallel clines in *D. simulans* populations from along the east coast of Australia that reflect the action of selection and not neutral processes. We know that temperature is a significant selective agent for *Drosophila* (Huey & Pascual, 2009; Rego *et al.*, 2010; Kellermann *et al.*, 2012), and the observed clinal patterns in heat tolerance in *D. simulans* (van Heerwaarden *et al.*, 2012) reflect this. The parallel clinal patterns suggest that the three traits provide the same insight into the adaptive divergence, and thus adaptive capacity, for heat tolerance. However, whether these parallel clinal patterns are the result of independent selection acting on all three measures of heat tolerance, or reflect a shared genetic basis, remains unknown. We therefore performed a half-sib–full-sib breeding design to empirically assess the additive genetic variance for, and additive genetic covariances between, all three measures of heat tolerance. This allowed us to test whether the predictions arising from previous work in *D. melanogaster*, where univariate estimates of adaptive capacity suggest a limit for adapting to the gradual increases in temperature that are commonly experienced in nature, hold true across species when an explicitly multivariate perspective is taken.

Materials and methods

Experimental stocks and data collection

D. simulans was collected from Coffs Harbour, a mid-latitude (latitude 30.30°S, longitude 153.12°E) site along the east coast of Australia, using banana baits in February 2010. Fifty field-inseminated females were collected and used to establish iso-female lines in the laboratory at 25 °C under a 12 : 12 hours light : dark cycle at 95–100% humidity. Species identification was confirmed in the F1 generation by examining males from each iso-female line, to ensure all lines were in fact *D. simulans*. These iso-female lines were then allowed an additional generation in the laboratory to ensure large population sizes in each line prior to setting up a mass-bred population. In the second generation after collection (F2), a mass-bred population was founded with ten males and ten females from each of the 50 iso-female lines. This mass-bred population was kept at 25 °C under a 12 : 12 hours light : dark cycle at 95–100% humidity in 3 × 250-mL bottles containing 60 mL of potato, yeast and sucrose media. Densities were approximately 300–350 flies per bottle to ensure a census population size of 900 + individuals.

After five generations of mass rearing, we used a paternal half-sibling breeding design to estimate the additive genetic variance underlying heat tolerance. The parents of the focal flies were reared at controlled densities of 40 eggs per vial, and were collected within 6 h of emergence. Virgin females and males were separated using CO_2 anaesthesia and held in separate vials according to sex, at a density of approximately 20–30 individuals per vial until they were 4-days old. A total of 150 virgin males (sires) were randomly selected from all holding vials. Each sire was placed in a vial containing 6 mL of food media and *ad libitum* live yeast, with three virgin females (dams) and left to mate for 3 days. After this time, each dam was placed individually in a separate vial and allowed to lay eggs for 6–8 h, then moved to a fresh vial and allowed to lay eggs for a further 6 h. This was done to control larval density to no more than 20 larvae per vial.

Generation 6 individuals – the focal offspring – were collected within 1 day of emerging in vials and held together in a fresh food vial for a further 48 h to ensure that females were mated. After 48 h, females were separated using CO_2 anaesthesia and allowed to recover for a further 48 h. Two females from each vial were measured for each heat-tolerance assay (for a total of 96–98 sires, each mated to three dams, with four offspring [minimum of two] per dam measured for each assay of heat tolerance). Flies used in the heat-tolerance assays were 5- to 6-days old. The half-sibling breeding design was performed at 25 °C under a 12 : 12 hours light : dark cycle at 95–100% humidity.

Heat-tolerance assays

Basal and hardened static heat-knockdown time

Females were placed individually in 5-mL glass vials, and exposed acutely to 38.5 °C by immersion in a pre-heated recirculating water bath. The hardening treatment involved exposure of flies to 35 °C for 30 min followed by recovery at 25 °C for 3 h prior to the knockdown assay being performed (van Heerwaarden *et al.*, 2012). Basal and hardened flies were tested simultaneously. Heat-knockdown time was scored as the time taken for all flies to be knocked down and immobilized. Heat-knockdown time was assessed over 2 days, with six runs performed each day. The same two people (observers) performed all of the heat-knockdown assays across all runs.

Ramping (dynamic) heat-knockdown time and temperature

Individual females were placed in 5-mL glass vials, which were submerged into a water bath heated to 28 °C. The temperature was increased gradually by 0.06 °C min^{-1}, which is representative of maximal rates of temperature increase in south-eastern Australia (van Heerwaarden *et al.*, 2012) and other parts of the world (Nyamukondiwa & Terblanche, 2010; Terblanche *et al.*, 2011). A data logger (Maxim Integrated i-button DS1923, Maxim Integrated Products, Dallas, TX, USA) was submerged into the heated water bath (along with the flies) to record the temperature of the heated water

bath throughout the experiment. Resistance was scored as the time taken for all flies to be knocked down, ensuring that all three traits were measured on the same scale. The ramping assays were performed over 2 days with two runs per day. The same two people (observers) performed all of the heat-knockdown assays across all runs.

Estimating the additive genetic variance covariance matrix, G

Our data were generated from a standard paternal half-sibling breeding design (Lynch & Walsh, 1998). The mixed model used to analyse the data was

$$y = \alpha + \mathbf{XB} + \mathbf{Z}_s\delta_s + \mathbf{Z}_d\delta_d + \epsilon, \quad (1)$$

where **X** is the design matrix for the fixed effect of run, **B,** and $\mathbf{Z}_s$ and $\mathbf{Z}_d$ are the design matrices for the random effects of sire and dam respectively. The total phenotypic variance (σ^2_P) for the breeding design for the purpose of estimating genetic parameters was represented by

$$\sigma^2_P = \sigma^2_S + \sigma^2_D + \sigma^2_W \quad (2)$$

where σ^2_S, σ^2_D, σ^2_W, are the sire, dam and within-group-level variance components respectively. Variance and covariance components were estimated using restricted maximum likelihood implemented using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). As we used a half-sib–full-sib breeding design, the sire variance, σ^2_S, is one-fourth of the additive genetic variance (V_A) (Falconer & Mackay, 1996; McGuigan *et al.*, 2011). Thus, to estimate V_A, we multiplied the sire variance by 4.

It has recently been suggested that observer error will affect the estimation of variance components for traits like heat-knockdown time (Castaneda *et al.*, 2012). Castaneda *et al.* (2012) suggest that multiple measurements for every individual be taken, and that repeatability statistics be reported for thermotolerance. This is not feasible in experiments such as those described here. Instead, we checked for a significant effect of observer on the phenotypic variance of all three measures of heat-knockdown time prior to estimating the variance components using Levene's test for equal variances. Observer had a significant effect on the variance of ramping heat-knockdown time ($F_{1,1092} = 8.30$, $P < 0.05$), but not static basal or hardened knockdown time (not shown). To ensure that this did not bias estimates of the variance and covariance components, we variance standardized all the heat-knockdown time data by observer prior to the analyses described below. Run did not affect the variances (not shown), so was treated as a fixed effect in the model described above.

The additive genetic variance for each trait was first estimated using a univariate model. Log likelihood ratio tests were performed, where the final model for each trait was compared to a model specifying σ^2_S to be zero, to determine whether levels of additive genetic variance for each trait were significantly different from zero (Littell *et al.*, 1996; Simonsen & Stinchcombe, 2010; McGuigan *et al.*, 2011). We then estimated the unconstrained **G** matrix. In both cases, the variance at both the sire, δ_s, and the dam, δ_d, levels were modelled using an unstructured covariance matrix. The additive genetic variance and covariance components of **G** were individually tested for significance from zero by performing log likelihood ratio tests where the final models for each trait were compared to models specifying σ^2_S and the sire-level covariances (COV_S) to be zero (Littell *et al.*, 1996; Simonsen & Stinchcombe, 2010; McGuigan *et al.*, 2011).

Dimensions of G

To examine the distribution of genetic variance in multivariate space, two complimentary approaches were utilized to estimate the number of dimensions of **G.**

Eigen analysis of G – Estimating $\mathbf{g_{max}}$

To determine how many genetically independent traits (eigenvectors) were represented by the original traits (phenotypes) actually measured, and how much genetic variance (eigenvalues) was associated with each independent set of eigenvectors, eigen analysis of the unconstrained additive genetic variance covariance matrix, **G**, was performed using the matrix analysis option implemented in the Microsoft Excel add-in PopTools (Hood, 2010). The eigenvector with the largest eigenvalue ($\mathbf{g}_{max}$, Schluter, 1996) is the vector explaining most of the additive genetic variance in the **G** matrix.

Kirkpatrick's dimensionality approach

We also used the approach outlined by Kirkpatrick (2009) to further explore the dimensions of the **G** matrix for the three heat traits, which strictly considers the geometry of **G** without regard to the direction of selection and the predicted response. This method determines the effective number of dimensions, n_D, in a **G** matrix by measuring whether there is an even distribution of genetic variation explained by all the eigenvalues of the **G** matrix estimated from the unconstrained model. If most of the genetic variation occurs in the first one or two dimensions, that matrix is ill conditioned and will permit evolution in only a limited number of dimensions. Kirkpatrick suggests measuring n_D as the sum of the eigenvalues of **G** divided by the largest eigenvalue. If n_D is close to 1, most of the genetic variation in **G** is explained by the first and

largest eigenvalue, and the matrix has an effective dimension of 1.

Additive genetic correlations between heat traits

To complement the multivariate methods described above, we estimated the additive genetic correlation between all three traits examined using the relationship

$$r_{S(1,2)} = \frac{\mathrm{Cov}_{S(1,2)}}{\sqrt{\sigma^2_{S1} \times \sigma^2_{S2}}} \quad (3)$$

using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA), where $\mathrm{Cov}_{S(1,2)}$ is the sire-level additive genetic covariance between traits 1 and 2, and σ^2_{S1} and σ^2_{S2} are the sire-level variance components for traits 1 and 2 (Lynch & Walsh, 1998). Log likelihood ratio tests were used to test whether any of the additive genetic correlations were significantly different from both zero and one (Littell *et al.*, 1996; Simonsen & Stinchcombe, 2010).

Univariate measures of evolvability

To directly assess the extent to which univariate measures of evolvability for heat tolerance reflected the multivariate analyses described above, we also estimated the narrow-sense heritability for each trait. Narrow-sense heritability for each trait was estimated as the additive genetic variance (V_A) divided by the total phenotypic variance (V_P) (Falconer & Mackay, 1996; Lynch & Walsh, 1998). Finally, the evolvability, I_A, of each trait was estimated as the additive genetic variance divided by the square of the trait mean following Hansen *et al.* (2011), as its numerical estimate can be interpreted as the per cent change in a trait under a unit strength of selection.

Results

Genetic variation and covariation for heat tolerance

We detected significant levels of additive genetic variance for all three measures of heat tolerance (Table 1). Additive genetic covariances between basal and hardened knockdown time were positive and significantly different from zero. The covariance between ramping heat-knockdown time and basal heat-knockdown time was marginally not signficantly different from zero, while the covariance between ramping and hardened heat-knockdown time was nonsignificant (Table 1).

One additive genetic correlation was significantly different from zero (Table 1). A significant positive genetic correlation was found between basal and hardened heat-knockdown time(Table 1). The genetic correlation between basal and hardened heat-knockdown time was significantly different from zero, but not one, implying that both traits will show correlated evolutionary responses to selection pressures. The genetic correlation between basal and ramping heat-knockdown time was positive, but marginally not significantly different from zero (Table 1).

Table 1 Additive genetic variance and covariance matrix (**G**) estimated from the model with unconstrained sire-level variances and covariances, and additive genetic correlations. Additive genetic variances on the diagonal, additive genetic covariances above the diagonal, additive genetic correlations below the diagonal. Basal = static basal heat-knockdown time; Hardened = static hardened heat-knockdown time; Ramping = ramping (dynamic) heat-knockdown time. Estimates based on raw data variance standardized by observer, multiplied by 100.

	Basal	Hardened	Ramping
Basal	6.434*	8.279*	5.202†
Hardened	0.768‡	18.069*	0.714
Ramping	0.511§	0.042	16.095*

*$P < 0.05$ for log likelihood ratio test of significant difference from zero.
†$P = 0.074$ for log likelihood ratio test of significant difference from zero.
‡$P < 0.05$ for log likelihood ratio test of significant difference from zero.
§$P = 0.074$ for log likelihood ratio test of significant difference from zero.

Eigen analysis of G – Estimating $\mathbf{g}_{max}$

The eigen analysis revealed that the genetic variance in **G** was distributed in two dimensions (Table 2). The two eigenvectors of **G** accounted for 97.8% of the total additive genetic variance of **G**. The leading eigenvector ($\mathbf{g}_{max}$) accounted for 58.44% of the variance in **G**. Basal, hardened and ramping heat-knockdown time all loaded positively to $\mathbf{g}_{max}$. Hardened heat-knockdown time made the largest contribution to this vector (Table 2). Basal and ramping heat-knockdown time

Table 2 Eigen analysis of genetic variation for all traits examined. Trait loadings on eigenvectors of the unconstrained sire-level additive genetic variance–covariance matrix (**G**), the additive genetic variance, V_A, (eigenvalue) associated with each eigenvector and the percentage of the total additive genetic ($\%V_A$) variance explained by each eigenvector. Basal = static basal heat-knockdown time; Hardened = static hardened heat-knockdown time; Ramping = ramping (dynamic) heat-knockdown time.

	$\mathbf{g}_{max}$	$\mathbf{g}_2$
V_A	23.726	15.983
$\%V_A$ total	58.44%	39.37%
Basal	0.4912	0.0486
Hardened	0.7702	−0.4906
Ramping	0.4069	0.8700

loaded positively, and hardened knockdown time negatively, onto the second eigenvector of **G** ($\mathbf{g}_2$), with the largest contribution coming from ramping heat-knockdown time.

Dimensionality of G

Using Kirkpatrick's (2009) method, we estimated $n_D = 1.7$. Thus, **G** has an effective dimension closer to 2. This is consistent with the eigen analysis of the **G** matrix (Table 2), which indicates that the first two eigenvectors account for almost all genetic variation in **G**.

Univariate mesasures of evolvability

Narrow-sense heritability estimates were significant for all three traits examined (Table 3), despite the environmental variance being larger for ramping heat-knockdown time, driven by the fact that all three traits had estimates of additive genetic variance that were significantly greater than zero. The relatively lower evolvability estimate for ramping heat-knockdown time suggests that the potential rate of univariate evolutionary change in this trait is less than for either static basal and hardened heat knockdown (Table 3).

Discussion

The expectation of ongoing selection for increased heat tolerance under climate change has renewed interest in the extent to which adaptation to thermal stress will be constrained by low levels of additive genetic variance (Mitchell & Hoffmann, 2010; Kellermann *et al.*, 2012).

It has been argued that different measures of heat tolerance may influence estimates of heritability, evolvability and inferences about an organism's ability to adapt to stressful environmental conditions (Chown *et al.*, 2009; Mitchell & Hoffmann, 2010; Rezende *et al.*, 2011). Specifically, it has been suggested that the heritability of upper thermal limits measured using ramping assays will be reduced largely due to an increase in the environmental variance (Chown *et al.*, 2009; Rezende *et al.*, 2011), although reductions in the additive genetic variance might also contribute to reduced estimates of heritability under ramping conditions (Rezende *et al.*, 2011). However, current debate about how best to predict and understand adaptive capacity for upper thermal limits has occurred within a univariate framework (Chown *et al.*, 2009; Mitchell & Hoffmann, 2010; Rezende *et al.*, 2011; Santos *et al.*, 2011), and ignores the fact that the ability of a population to respond to selection for increasing heat tolerance will be determined by the patterns of genetic variation and covariation in the traits under selection.

The motivation of our study was to take a multivariate approach to estimating adaptive capacity for upper thermal limits, and in doing so, determine the extent to which adaptive capacity differs across three different, but commonly used, measures of heat tolerance in *D. simulans*. We first showed significant additive genetic variance for all three traits examined. We then determined that the additive genetic variance–covariance matrix, **G**, was of reduced rank, with two eigenvectors explaining close to 100% of the additive genetic variance in **G** ($\mathbf{g}_{max}$ 58%, $\mathbf{g}_2$ 39%). Hardened and basal static heat-knockdown time made the largest contribution to $\mathbf{g}_{max}$, followed by ramping heat-knockdown time, suggesting that selection for increased heat tolerance (in the direction of $\mathbf{g}_{max}$) should result in evolutionary increases in heat tolerance in all three traits.

We then demonstrated that two of the three traits also have the potential to evolve via correlated responses to selection for heat tolerance. Static basal and hardened heat-knockdown time were positively genetically correlated to each other, while the genetic correlation between ramping and static basal heat-knockdown time was marginally nonsignificant. Recent interspecific comparisons in *Drosophila* (Nyamukondiwa *et al.*, 2011) also reveal a positive association between static basal and hardened heat tolerance, although an intraspecific study in *D. melanogaster* did not (Sgrò *et al.*, 2010). Although Mitchell & Hoffmann (2010) suggested that static basal and ramping heat-knockdown time might be correlated based on interspecific correlations between the two traits, our data indicate that adult responses to static and ramping thermal stress are largely genetically independent, which is consistent with previous work (Sgrò *et al.*,

Table 3 Mean heat-knockdown time (min), additive genetic variance (V_A), environmental variance (V_E), phenotypic variance (V_P) and narrow-sense heritability (h^2), and evolvability (I_A) for basal, static hardened and ramping heat-knockdown time. N = sample size. Basal = static basal heat-knockdown time; Hardened = static hardened heat-knockdown time; Ramping = ramping (dynamic) heat-knockdown time.

Trait	Mean ± SE	V_A ± SE	V_E ± SE	V_P ± SE	h^2 ± SE	I_A	N
Basal	13.489 ± 0.112	6.434 ± 2.893*	20.299 ± 0.626	20.299 ± 1.321	0.241 ± 0.028†	0.0348	1130
Hardened	15.700 ± 0.149	18.069 ± 6.015*	18.442 ± 0.973	36.511 ± 1.868	0.495 ± 0.032†	0.0733	1110
Ramping	159.585 ± 0.203	16.095 ± 9.252*	67.947 ± 1.978	84.042 ± 4.274	0.192 ± 0.007†	0.0006	1094

*$P < 0.05$ for log likelihood ratio test of significant difference from zero.
†$P < 0.05$.

2010). The absence of a significant genetic correlation between ramping and static hardened heat resistance is somewhat surprising. It has previously been suggested that responses to ramping heat stress likely involve hardening responses (Chown *et al.*, 2009; Sgrò *et al.*, 2010; Rezende *et al.*, 2011). However, we show for the first time that ramping heat-knockdown time and static hardened heat-knockdown time are genetically independent of each other at least in the population of *D. simulans* examined here. These results indicate that the parallel clines observed for all three traits in populations of *D. simulans* from eastern Australia (van Heerwaarden *et al.*, 2012) are likely the result of both correlated and direct responses to selection.

On the basis of nonsignificant estimates of additive genetic variance and narrow-sense heritability, Mitchell & Hoffmann (2010) concluded that there was limited potential for *D. melanogaster* to adapt in response to the gradual increases in temperature that are frequently observed in nature. Our results contrast with these predictions in two ways. Firstly, our narrow-sense heritability and additive genetic variance estimates for ramping heat knockdown were in fact significantly different from zero, although its evolvability was relatively lower than that for static basal and hardened heat-knockdown time. Second, and more importantly, our multivariate analysis indicated that multiple measures of heat tolerance will contribute to evolutionary responses to selection. The results of Mitchell & Hoffmann (2010) seem to be at odds with the parallel linear clines in static and ramping female heat tolerance reported for *D. melanogaster* populations from eastern Australia (Sgrò *et al.*, 2010). Whether the cline in ramping heat-knockdown time in *D. melanogaster* is the result of correlated responses to selection on an unmeasured trait is not known. Additive genetic covariances between ramping heat-knockdown time and other traits have not been examined in *D. melanogaster*.

A true empirical test of the extent to which the evolution of heat tolerance in *D. simulans* may or may not be constrained by genetic variances or covariances, however, requires both an estimate of the additive genetic variance–covariance matrix (**G**) and the vector of directional selection gradient, $\boldsymbol{\beta}$, for all traits (Lynch & Walsh, 1998). While we have estimated the former, we do not have direct estimates of $\boldsymbol{\beta}$ for any of the traits examined. We can only infer the role of natural selection from clinal studies of *D. simulans* from eastern Australia that demonstrate parallel clines in all three heat-tolerance traits (van Heerwaarden *et al.*, 2012) as well as linear clines in starvation resistance and body (wing) size (Arthur *et al.*, 2008; van Heerwaarden & Sgrò, 2011) that have been shown to result from selection rather than genetic drift. Whether ongoing selection for increased heat tolerance in *D. simulans* will result in unconstrained long-term evolutionary responses, or whether the multivariate genetic variance for upper thermal limits might be exhausted, resulting in a selection response plateau (Gilchrist & Huey, 1999) remains to be investigated. Further studies that take an explicitly multivariate perspective to this question are required.

In addition, while it has been suggested that evolutionary responses of upper thermal limits to increasing temperatures will not be sufficient to overcome the threat of climate warming (e.g. Kellermann *et al.*, 2012; Overgaard *et al.*, 2012), the role that changing environmental conditions might have on the evolution of upper thermal limits has not yet been considered. The additive genetic variance can change with environmental conditions (e.g. Sgrò & Hoffmann, 1998; Hoffmann & Merila, 1999; Charmantier & Garant, 2005; McGuigan & Sgrò, 2009), meaning that the potential for traits to respond to selection may also change as the environment changes. This can be extended to a multivariate context, whereby the additive genetic variances for, and additive genetic covariances between, traits may change as the environment changes. In addition, it is also possible that the strength of selection acting on this additive genetic variance–covariance may also change as the environment changes (Husby *et al.*, 2011). This is significant because the effect of environmental variation on both additive genetic variances and covariances and the strength of selection may affect the evolutionary dynamics of natural populations (Husby *et al.*, 2011). For instance, Wilson *et al.* (2006) showed that the strength of selection on body weight in Soay sheep in a given year was negatively correlated with the expression of total genetic variance, suggesting a possible constraint on the evolution of body weight in this population. In contrast, studying timing of breeding in a wild population of great tits, Husby *et al.* (2011) showed that in years where spring temperatures were highest, selection was strongest and the additive genetic variance was also highest, suggesting that the speed of microevolutionary change could in fact be increased by changing environmental conditions. The predicted response to selection was also highly temperature dependent. Indeed, Husby *et al.* (2011) showed that by not incorporating environmental dependence of the expression of genetic variance and strength of selection, the predicted response to selection may be underestimated by up to 20%. The findings of Husby *et al.* (2011) are significant because, if applicable more broadly, they reveal a mechanism that could potentially increase the speed of adaptation to climate change. While Bubliy *et al.* (2012) found no effect of different levels of humidity on the additive genetic variance for heat tolerance in *D. melanogaster*, the levels of humidity were extreme, and not necessarily reflective of natural conditions, and their study took a univariate approach. Further studies are required to determine whether the dependence of additive genetic variances

and covariances and strength of selection on environmental conditions might influence the evolution of upper thermal limits more broadly. Failing to consider the environmental dependence of the expression of genetic variance and covariance and strength of selection, and environmentally dependent associations between the two, may limit our understanding of microevolutionary responses to climate change, and lead to inaccurate predictions about extinction risk (Husby *et al.* (2011).

Finally, it has been argued that estimates of upper thermal limits obtained using ramping rates such as those used in this study will be confounded by the simultaneous effects of resource depletion through desiccation and starvation and hardening/acclimation on organisms (Rezende *et al.*, 2011; Santos *et al.*, 2011). Recent work (Overgaard *et al.*, 2012) indicates that neither starvation nor desiccation stress confound estimates of upper thermal limits using ramping rates $\geq$ 0.06 °C min^{-1}. Furthermore, parallel clines in static and dynamic measures of heat resistance in both adults and larvae of *D. simulans* (van Heerwaarden *et al.*, 2012) and adults of *D. melanogaster* (Sgrò *et al.*, 2010) from eastern Australia suggest that this is not the case. In addition, it is unlikely that laboratory adaptation has influenced the results of this study, as it has previously been shown that heat-knockdown time was not influenced by time spent in the laboratory in *D. birchii* (Griffiths *et al.*, 2005).

In conclusion, our multivariate analysis showed that static and dynamic measures of thermal tolerance provide similar insight into the potential for upper thermal limits to evolve in response to selection, even though they are in effect genetically independent. Our **G** matrix analysis showed that all three traits will contribute to responses to selection for increased heat tolerance, and that low levels of additive genetic variance for, or covariances between, the traits will not constrain the evolution of heat tolerance in response to selection expected to occur under climate warming in the population of *D. simulans* examined. Further empirical studies that take an explicitly multivariate perspective to the evolution of thermal tolerance, which not only examine populations sampled throughout a species' range but that also consider multiple environments (Husby *et al.*, 2011) and environmental stressors at the same time (Clusella-Trullas *et al.*, 2011; Terblanche *et al.*, 2011; Bubliy *et al.*, 2012; Kellermann *et al.*, 2012), are needed.

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References

Arthur, A.L., Weeks, A.R. & Sgrò, C.M. 2008. Investigating latitudinal clines for life history and stress resistance traits in *Drosophila simulans* from eastern Australia. *J. Evol. Biol.* **21**: 1470–1479.

Bennett, A.F. & Lenski, R.E. 1993. Evolutionary adaptation to temperature. 2. Thermal niches of experimental lines of Escherichia coli. *Evolution* **47**: 1–12.

Berrigan, D. 2000. Correlations between measures of thermal stress resistance within and between species. *Oikos* **89**: 301–304.

Blows, M.W. & Hoffmann, A.A. 2005. A reassessment of genetic limits to evolutionary change. *Ecology* **86**: 1371–1384.

Bubliy, O.A., Imasheva, A.G. & Loeschcke, V. 1998. Selection for knockdown resistance to heat in *Drosophila melanogaster* at high and low larval densities. *Evolution* **52**: 619–625.

Bubliy, O.A., Kristensen, T.N., Kellermann, V. & Loeschcke, V. 2012. Humidity affects genetic architecture of heat resistance in *Drosophila melanogaster*. *J. Evol. Biol.* **25**: 1180–1188.

Castaneda, L.E., Calabria, G., Betancourt, L.A., Rezende, E.L. & Santos, M. 2012. Measurement error in heat tolerance assays. *J. Therm. Biol* **37**: 432–437.

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

Charmantier, A. & Garant, D. 2005. Environmental quality and evolutionary potential: lessons from wild populations. *Proc. R. Soc. B Biol. Sci.* **272**: 1415–1425.

Chown, S.L., Jumbam, K.R., Sorensen, J.G. & Terblanche, J.S. 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodology. *Funct. Ecol.* **23**: 133–140.

Chown, S.L., Hoffmann, A.A., Kristensen, T.N., Angilletta, M.J., Stenseth, N.C. & Pertoldi, C. 2010. Adapting to climate change: a perspective from evolutionary physiology. *Clim. Res.* **43**: 3–15.

Clusella-Trullas, S., Blackburn, T.M. & Chown, S.L. 2011. Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *Am. Nat.* **177**: 738–751.

Colwell, R.K., Brehm, G., Cardelus, C.L., Gilman, A.C. & Longino, J.T. 2008. Global warming, elevational range shifts, and lowland biotic attrition in the wet tropics. *Science* **322**: 258–261.

Cossins, A. & Bowler, K. 1987. *Temperature biology of animals*. pp. 339. Chapman and Hall, London.

Coyne, J.A., Bundgaard, J. & Prout, T. 1983. Geographic variation of tolerance to environmental stress in *Drosophila pseudoobscura*. *Am. Nat.* **122**: 474–488.

Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. *et al.* 2008. Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. USA* **105**: 6668–6672.

Dillon, M.E., Wang, G. & Huey, R.B. 2010. Global metabolic impacts of recent climate warming. *Nature* **467**: 704–706.

Doyle, C.M., Leberg, P.L. & Klerks, P.L. 2011. Heritability of heat tolerance in a small livebearing fish, *Heterandria formosa*. *Ecotoxicology* **20**: 535–542.

Duarte, H., Tejedo, M., Katzenberger, M., Marangoni, F., Baldo, D., Beltran, J.F. *et al.* 2012. Can amphibians take the

heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. *Glob. Change Biol.* **18**: 412–421.

Elderkin, C.L., Stoeckel, J.A., Klerks, P.L. & Berg, D.J. 2004. Heritability of heat tolerance in zebra mussel veligers. *J. Gt. Lakes Res.* **30**: 360–366.

Falconer, D.S. & Mackay, T.F.C. 1996. *Introduction to Quantitative Genetics*. Pearson Education Limited, Harlow.

Folk, D.G., Hoekstra, L.A. & Gilchrist, G.W. 2007. Critical thermal maxima in knockdown-selected *Drosophila*: are thermal endpoints correlated? *J. Exp. Biol.* **210**: 2649–2656.

Gilchrist, G.W. & Huey, R.B. 1999. The direct response of *Drosophila melanogaster* to selection on knockdown temperature. *Heredity* **83**: 15–29.

Griffiths, J.A., Schiffer, M. & Hoffmann, A.A. 2005. Clinal variation and laboratory adaptation in the rainforest species *Drosophila birchii* for stress resistance, wing size, wing shape and development time. *J. Evol. Biol.* **18**: 213–222.

Hansen, T.F., Pelabon, C. & Houle, D. 2011. Heritability is not Evolvability. *Evol. Biol.* **38**: 258–277.

van Heerwaarden, B. & Sgrò, C.M. 2011. The effect of developmental temperature on the genetic architecture underlying size and thermal clines in *Drosophila melanoagster* and *D. simulans* from the east coast of Australia. *Evolution* **65**: 1048–1067.

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

Hoffmann, A.A. 2010. Physiological climatic limits in *Drosophila*: patterns and implications. *J. Exp. Biol.* **213**: 870–880.

Hoffmann, A.A. & Merila, J. 1999. Heritable variation under favourable and unfavourable conditions. *Trends Ecol. Evol.* **14**: 96–101.

Hoffmann, A.A., Dagher, H., Hercus, M. & Berrigan, D. 1997. Comparing different measures of heat resistance in selected lines of *Drosophila melanogaster*. *J. Insect Physiol.* **43**: 393–405.

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

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

Hood, G. M. 2010. PopTools version 3.2.5. Available at http://www.poptools.org.

Huey, R.B. & Pascual, M. 2009. Partial thermoregulatory compensation by a rapidly evolving invasive species along a latitudinal cline. *Ecology* **90**: 1715–1720.

Huey, R.B. & Tewksbury, J.J. 2009. Can behavior douse the fire of climate warming? *Proc. Natl Acad. Sci. USA* **106**: 3647–3648.

Huey, R.B., Deutsch, C.A., Tewksbury, J.J., Vitt, L.J., Hertz, P.E., Perez, H.J.A. *et al.* 2009. Why tropical forest lizards are vulnerable to climate warming. *Proc. R. Soc. B Biol. Sci.* **276**: 1939–1948.

Huey, R.B., Kearney, M.R., Krockenberger, A., Holtum, J.A.M., Jess, M. & Williams, S.E. 2012. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philos. T. R. Soc. B* **367**: 1665–1679.

Husby, A., Visser, M. & Kruuk, L. 2011. Speeding up microevolution: the effects of increasing temperature on selection and genetic variance in a wild bird population. *PLoS Biol.* **9**: e1000585.

Intergovernmental Panel on Climate Change. 2007. *Climate Change 2007: Synthesis Report*. (Intergovernmental Panel on Climate Change Geneva).

Jenkins, N.L. & Hoffmann, A.A. 1994. Genetic and maternal variation for heat resistance in *Drosophila* from the field. *Genetics* **137**: 783–789.

Jones, S.J., Mieszkowska, N. & Wethey, D.S. 2009. Linking thermal tolerances and biogeography: *Mytilus edulis* (L.) at its southern limit on the east coast of the United States. *Biol. Bull.* **217**: 73–85.

Jumbam, K.R., Jackson, S., Terblanche, J.S., McGeoch, M.A. & Chown, S.L. 2008. Acclimation effects on critical and lethal thermal limits of workers of the Argentine ant, *Linepithema humile*. *J. Insect Physiol.* **54**: 1008–1014.

Kearney, M., Shine, R. & Porter, W.P. 2009. The potential for behavioral thermoregulation to buffer "cold-blooded" animals against climate warming. *Proc. Natl. Acad. Sci. USA* **106**: 3835–3840.

Kellermann, V., Overgaard, J., Hoffman, A. A., Flojgaard, C., Svenning, J.-C. & Loeschcke, V. 2012. Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. USA* **109**: 16228–16233.

Kelly, M.W., Sanford, E. & Grosberg, R.K. 2012. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* **279**: 349–356.

Kelty, J.D. & Lee, R.E. 2001. Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *J. Exp. Biol.* **204**: 1659–1666.

Kirkpatrick, M. 2009. Patterns of quantitative genetic variation in multiple dimensions. *Genetica* **136**: 271–284.

Krebs, R.A. & Thompson, K.A. 2006. Direct and correlated effects of selection on flight after exposure to thermal stress in *Drosophila melanogaster*. *Genetica* **128**: 217–225.

Littell, R., Milliken, A., Stroup, W. & Wolfinger, R. 1996. *SAS System for Mixed Models*. SAS Institute Inc, Cary, NC.

Lynch, M. & Walsh, B. 1998. *Genetics and the Analysis of Quantitative Traits*. Sinauer Associates Inc, Sunderland.

McColl, G., Hoffman, A.A. & McKechnie, S. 1996. Response of two heat shock genes to selection for knockdown heat resistance in *Drosophila melanogaster*. *Genetics* **143**: 1615–1627.

McGuigan, K. & Sgrò, C.M. 2009. Evolutionary consequences of cryptic genetic variation. *Trends Ecol. Evol.* **24**: 305–311.

McGuigan, K., Nishimura, N., Currey, M., Hurwit, D. & Cresko, W.A. 2011. Cryptic genetic variation and body size evolution in threespine stickleback. *Evolution* **65**: 1203–1211.

Mercader, R.J. & Scriber, J.M. 2008. Asymmetrical thermal constraints on the parapatric species boundaries of two widespread generalist butterflies. *Ecol. Entomol.* **33**: 537–545.

Mitchell, K.A. & Hoffmann, A.A. 2010. Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. *Funct. Ecol.* **24**: 694–700.

Neargarder, G., Dahlhoff, E.P. & Rank, N.E. 2003. Variation in thermal tolerance is linked to phosphoglucose isomerase genotype in a montane leaf beetle. *Funct. Ecol.* **17**: 213–221.

Nyamukondiwa, C. & Terblanche, J.S. 2010. Within-generation variation of critical thermal limits in adult Mediterranean and Natal fruit flies *Ceratitis capitata* and *Ceratitis rosa*: thermal history affects short-term responses to temperature. *Physiol. Entomol.* **35**: 255–264.

Nyamukondiwa, C., Terblanche, J.S., Marshall, K.E. & Sinclair, B.J. 2011. Basal cold but not heat tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). *J. Evol. Biol.* **24**: 1927–1938.

Overgaard, J., Sorensen, J.G., Petersen, S.O., Loeschcke, V. & Holmstrup, M. 2006. Reorganization of membrane lipids during fast and slow cold hardening in *Drosophila melanogaster*. *Physiol. Entomol.* **31**: 328–335.

Overgaard, J., Kristensen, T.N. & Sorensen, J. 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods. *PLoS ONE* **7**: e32758.

Parmesan, C. & Yohe, G. 2003. A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**: 37–42.

Rego, C., Balanya, J., Fragata, I., Matos, M., Rezende, E.L. & Santos, M. 2010. Clinal patterns of chromosomal inversion polymorphisms in *Drosophila subobscura* are partially associated with thermal preferences and heat stress resistance. *Evolution* **64**: 385–397.

Rezende, E.L., Tejedo, M. & Santos, M. 2011. Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. *Funct. Ecol.* **25**: 111–121.

Santos, M., Castaneda, L.E. & Rezende, E.L. 2011. Making sense of heat tolerance estimates in ectotherms: lessons from *Drosophila*. *Funct. Ecol.* **25**: 1169–1180.

Schluter, D. 1996. Adaptive radiation along genetic lines of least resistance. *Evolution* **50**: 1766–1774.

Sgrò, C.M. & Hoffmann, A.A. 1998. Effects of temperature extremes on genetic variances for life history traits in *Drosophila melanogaster* as determined from parent-offspring comparisons. *J. Evol. Biol.* **11**: 1–20.

Sgrò, C.M., Overgaard, J., Kristensen, T.N., Mitchell, K.A., Cockerell, F.E. & Hoffmann, A.A. 2010. A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from eastern Australia. *J. Evol. Biol.* **23**: 2484–2493.

Simonsen, A.K. & Stinchcombe, J.R. 2010. Quantifying evolutionary genetic constraints in the ivyleaf morning glory, *Ipomoea hederacea*. *Int. J. Plant Sci.* **171**: 972–986.

Sinervo, B., Mendez-de-la-Cruz, F., Miles, D.B., Heulin, B., Bastiaans, E., Cruz, M.V.S. *et al.* 2010. Erosion of lizard diversity by climate change and altered thermal niches. *Science* **328**: 894–899.

Sisodia, S. & Singh, B. N. 2010. Resistance to environmental stress in *Drosophila ananassae*: latitudinal variation and adaptation among populations. *J. Evol. Biol.* **23**: 1979–1988.

Somero, G.N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **213**: 912–920.

Sorensen, J.G., Dahlgaard, J. & Loeschcke, V. 2001. Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Funct. Ecol.* **15**: 289–296.

Sorensen, J.G., Kristensen, T.N., Kristensen, K.V. & Loeschcke, V. 2007. Sex specific effects of heat induced hormesis in Hsf-deficient *Drosophila melanogaster*. *Exp. Gerontol.* **42**: 1123–1129.

Stillman, J.H. 2002. Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, genus *Petrolisthes*. *Integr. Comp. Biol.* **42**: 790–796.

Stillman, J.H. & Somero, G.N. 2000. A comparative analysis of the upper thermal tolerance limits of eastern Pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. *Physiol. Biochem. Zool.* **73**: 200–208.

Terblanche, J.S., Deere, J.A., Clusella-Trullas, S., Janion, C. & Chown, S.L. 2007. Critical thermal limits depend on methodological context. *Proc. R. Soc. B Biol. Sci.* **274**: 2935–2942.

Terblanche, J.S., Hoffmann, A.A., Mitchell, K.A., Rako, L., le Roux, P.C. & Chown, S.L. 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. *J. Exp. Biol.* **214**: 3713–3725.

Tomanek, L. & Somero, G.N. 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J. Exp. Biol.* **202**: 2925–2936.

Willett, C.S. 2010. Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution* **64**: 2521–2534.

Wilson, A.J., Pemberton, J.M., Pilkington, J.G., Coltman, D.W., Mifsud, D.V., Clutton-Brock, T.H. *et al.* 2006. Environmental coupling of selection and heritability limits evolution. *PLoS Biol.* **4**: 1270–1275.

Winne, C.T. & Keck, M.B. 2005. Intraspecific differences in thermal tolerance of the diamondback watersnake (*Nerodia rhombifer*): effects of ontogeny, latitude, and sex. *Comp. Biochem. Phys. A* **140**: 141–149.

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