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Stress tolerance in a novel system: Genetic and environmental sources of (co)variation for cold tolerance in the butterfly *Eurema smilax*

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Abstract The physiological ability to survive climatic extremes, such as low temperature, is a major determinant of species distribution. Research suggests that tropically restricted insect populations may possess low to zero variation in stress tolerance, thereby limiting any potential to adapt to colder climates. This paradigm derives largely from contrasts among *Drosophila* populations and species along the tropical–temperate cline of eastern Australia. Butterfly groups, such as the variously distributed representatives of the genus *Eurema*, offer opportunities to test the taxonomic breadth of this paradigm. We contribute here by investigating plasticity, repeatability and heritability (h^2) for cold tolerance in *Eurema smilax*. This continentally widespread species (extending from the Torres Strait to the south coast of Victoria) offers an important comparative basis for evaluating stress tolerance in geographically restricted congenerics. We reared two generations of *E. smilax* under laboratory conditions and measured recovery from a chill-coma assay, which is one of the commonly used methods for characterizing adult cold stress tolerance. Trials on F2s conducted over three consecutive days revealed individual repeatability ($r = 0.405$). However, recovery time decreased systematically across trials, which is characteristic of a phenotypically plastic 'hardening' response to prior cold exposure. Generalized linear modelling, wherein genetic variance was estimated via an 'animal model' approach, indicated no difference between sexes and no effect of body size, but a significant additive genetic term, corresponding to a heritability estimate of $h^2 = 0.414 \pm 0.100$. These data suggest significant adaptive potential for cold tolerance in *E. smilax* but show that individuals may also respond directly to extremes of cold via phenotypic plasticity. This indicates the potential to adapt to varied thermal extremes, which would be expected for a broadly distributed species that is resilient to climate change.

Key words: climate change, hardening response, heritability, plasticity, species distribution.

INTRODUCTION

The ability to survive at the extremes of local ambient temperature – the property known as thermal tolerance (Terblanche *et al.* 2011) – is an important determinant of contemporary biogeography (Gaston 2009). Given the expectations for global climate change, this parameter is likewise considered a key driver of future variation in species distribution (Wilson *et al.* 2005; Hickling *et al.* 2006). In many cases, thermal tolerance will influence distributions on ecological timescales, simply via setting the geographic limits to population viability (Kellermann *et al.* 2009; Régnière *et al.* 2012). In rapidly evolving organisms, however, there is a potential for thermal tolerance itself to evolve under changed thermal regimes (Gienapp *et al.* 2008; Hoffmann & Sgro 2011). Rather than (or in addition to) modifying their distribution, such species may possess the ability to adapt to changing climates. The rate and extent of adaptive evolution will depend upon a range of factors, including effective population size, generation time and the rate of environmental change (the equivalent of selection differential) (Lynch & Lande 1993; Charmantier *et al.* 2008). As a key component of realized heritability (Kellermann *et al.* 2006; Sgro *et al.* 2010; van Heerwaarden & Sgro 2013), levels of additive genetic variation will be crucial to adaptation as well as phenotypic plasticity. Understanding the sources of population variance in stress traits such as thermal tolerance may greatly inform predictions of biological responses to future climate change (Hoffmann & Willi 2008; Gilman *et al.* 2010; Hoffmann & Sgro 2011; Bellard *et al.* 2012; Régnière *et al.* 2012).

Ectothermic groups such as insects are considered particularly sensitive to localized climatic variation (Deutsch *et al.* 2008; Clusella-Trullas *et al.* 2011). Interestingly, because of their rapid generation times and large effective population sizes, many of these species also possess a high potential for adaptive evolution (Hoffmann & Sgro 2011). Compared with endotherms, their heightened thermal sensitivity arises from the stronger influence of ambient temperature upon physiological processes and baseline metabolic rate (Gillooly *et al.* 2001). Temperature is known to strongly influence insect development, growth and other key determinants of lifetime fitness (Jones *et al.* 1987; Sgro & Hoffmann 1998; Karl & Fischer 2009). Within insects, there are

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varied adaptations for dealing with thermal variation within and among their operational environments, including strategic basking and heat avoidance behaviours, often coupled with surface morphologies that influence heat transfer rates (e.g. colouration). Nevertheless, insects remain most active within a narrow range of temperatures. This range varies between species and populations because of adaptations to local climate and seasonality.

Adaptive evolution may sustain longer-term population responses to environmental change, but shorter-term responses will be determined by plasticity and phenotypic variation in thermal tolerance (Ayrinhac *et al.* 2004; Gienapp *et al.* 2008). Plasticity in thermal tolerance can be induced by exposure to the environment itself, such as acclimatization due to conditions experienced in juvenile stages (Larsen & Lee 1994; Gibert & Huey 2001; Ayrinhac *et al.* 2004; Zeilstra & Fischer 2005; Fischer *et al.* 2010) or through short-term exposure of adults to thermal extremes (i.e. so-called 'hardening' responses; Larsen & Lee 1994; Hoffmann *et al.* 2003b; Fischer *et al.* 2010). All forms of variation may potentially buffer or accentuate the effects of short-term environmental variation at the population level. However, for most insect groups (and indeed, more broadly in evolutionary theory; Ghalambor *et al.* 2007), how plasticity might influence longer-term adaptation is presently unclear.

Research on insect thermal tolerance can focus on either temperate species (Larsen & Lee 1994; Ayrinhac *et al.* 2004; Overgaard *et al.* 2011; Logan *et al.* 2012) or tropical species (Kellermann *et al.* 2006; Fischer *et al.* 2010; Dierks *et al.* 2012b), while fewer studies have contrasted temperate *versus* tropical species (Addo-Bediako *et al.* 2000; Deutsch *et al.* 2008; Sunday *et al.* 2011). A series of studies have compared temperate and tropical *Drosophila* species exclusively across the latitudinal gradient presented by the eastern Australian coastline (Gibert & Huey 2001; Sgro *et al.* 2010; Mitchell *et al.* 2011). The sum of work in *Drosophila* has increasingly revealed evidence for a notable biogeographic basis to stress tolerance, namely, that tropically restricted species exhibit reduced levels of phenotypic and genetic variation relative to their more broadly and/or temperately distributed counterparts (Kellermann *et al.* 2009; Mitchell *et al.* 2011). This trend is stronger for cold tolerance than heat and desiccation resistance owing to higher heritability and variance in cold tolerance (Kimura 2004; Kellermann *et al.* 2006; Blackburn *et al.* 2014). This work also offers an important case study for how contemporary genetic variation may inform adaptive potential, both as estimated via laboratory selection protocols (Hoffmann *et al.* 2003a) and as anticipated for wild populations (Hoffmann 2010; Sgro *et al.* 2010; Kellermann *et al.* 2012). Various evolutionary genetic hypotheses have been proposed for this tropical/temperate pattern of variance (e.g. DNA decay, whereby genetic variation is lost through stochastic factors in the absence of any selection). However, irrespective of its causal basis, the pattern implies that tropically restricted populations may possess intrinsically lower potential for adaptive change. This has potentially great significance because most global biodiversity resides in the tropics, which presents a clear need to address stress trait variation across a broader range of taxa. However, new heritability estimates of desiccation resistance in tropical *Drosophila* are much higher than old estimates that used unrealistic levels of desiccation stress, and this indicates a potential shift in the current paradigm (van Heerwaarden & Sgro 2013).

In this study, we aimed to explore the (co)variation of body size, sex and genetic ancestry of parents with cold tolerance in the small grass yellow butterfly, *Eurema smilax*. This is one of seven Australian *Eurema* species (Braby 2000). *Eurema* have varied distributions ranging from highly tropically restricted (*Eurema puella*), primarily tropical (*Eurema laeta*, *Eurema alitha* and *Eurema herla*) to tropical to sub-tropical (*Eurema hecabe* and *Eurema brigitta*) to continentally widespread (e.g. *E. smilax*) (Jones & Rienks 1987; Braby 2000). The group offers a useful empirical analogy to the *Drosophila* system, and efforts have begun to test associations between biogeography and adaptive potential in several *Eurema* species. Davis *et al.* (2014) recently reported differences in broad-sense heritability (H^2) of cold tolerance in three species (*E. laeta* [$H^2 = 0.131$], *E. hecabe* [$H^2 = 0.294$] and *E. smilax* [$H^2 = 0.327$]), indicating an increasing trend from the most restricted (*E. laeta*) to the most widespread species (*E. smilax*). Here, we expand the knowledge basis for *E. smilax* by using a larger multigenerational design to estimate (i) narrow-sense heritability (h^2) for chill-coma recovery and (ii) individual repeatability and phenotypic plasticity for this trait. We define plasticity in terms of sample-wide variation in absolute recovery time across trials.

METHODS

Specimen provenance and rearing protocols

This study was performed using the lab-reared F1 and F2 offspring deriving from F0 female *E. smilax* collected from Narrabri ($n = 19$; location: 30°32.45′S, 149°79.30′E) and Quirindi ($n = 7$; location: 31°51.73′S, 150°69.33′E) in March 2012. *Eurema smilax* is known to migrate over many hundreds of kilometres, with seasonal range expansions occurring on a scale of thousands of kilometres (Smithers 1983; Jones & Rienks 1987; Dingle *et al.* 1999; Braby 2000). This spans across cool temperate, sub-tropical and tropical climates of continental Australia (and with sporadic records for Tasmania; Braby 2000). Our two sampled locations are about 180 km apart in a contiguous breeding habitat. We anticipated high gene flow

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across this range, especially during the summer months (January–May).

Each F0 female was encouraged to oviposit onto a single cutting of the perennial herb *Neptunia gracilis* inside a plastic cup (90-mm diameter × 140 mm height). F1 larvae were counted 3–4 days post-hatching, and families with more than 12 individuals were split equally among two rearing cups to avoid overcrowding. Fresh plant cuttings were inserted into the cups every 2–3 days, or as otherwise required. Twice during their development, larvae were moved to fresh plants in clean cups using a fine paintbrush. Laboratory-rearing conditions consisted of a 14:10 L : D photoperiod (with light supplied by 400-W metal-halide lighting), 30–45% relative humidity and 24.0 ± 1.0 °C temperature. To increase growth and survival, second-instar larvae were relocated to a room set at 28.0 ± 1.0 °C during the photophase.

Once they emerge, adults were allowed to stand for >2 h prior to handling. We sexed each individual based on the presence/absence of a male-limited wing marking, measured forewing length (from apex to insertion) to the nearest 0.1 mm using digital callipers and wrote a unique identifying number on the ventral hindwing using a soft felt tip pen (Sharpie, Sanford, Braeside, Victoria, Australia). Individuals were then transferred to plastic specimen jars (40-mm diameter × 60-mm height) and provided *ad libitum* access to honey water (1:9 ratio) on a cotton wool pad anchored immediately beneath the lid of each jar.

After assessing individual cold tolerance (see later discussion), F1s were placed into a 600-mm^3 mating enclosure (aluminium-flywire construction). Lighting was provided by several 400-W metal-halide UV lights, situated 300 mm above the cage. Ambient temperature in the room was controlled to 28.0 ± 1.0 °C, and relative humidity ranged from 30% to 40%. The lights were kept on for 8–10 h day^{-1}. Given that the first copulation in *Eurema* butterflies normally lasts 30–40 min (Kemp 2008), cages were checked every 15–20 min to locate copulating pairs. Butterflies were only allowed to copulate once, such that all F2 families comprised full siblings with known dams and sires.

Assaying cold tolerance in insects

The minimum temperature at which ectotherms can no longer remain active and express normal cell function is known as their critical thermal minimum (CTM) (MacMillan & Sinclair 2011). Research into the CTM, which defines individual cold tolerance, has progressed via physiological and behavioural assays (Terblanche *et al.* 2011). The behavioural 'chill-coma' assay has been used widely to study variation in cold tolerance across different insect species and populations (Hoffmann *et al.* 2003b; Terblanche *et al.* 2011). For this assay, test subjects are placed at a temperature below their CTM, which elicits a semi-comatose, unresponsive state known as chill coma. The coma-like state results from the cessation of neural activity as ion gates become inactive, and cells reach oxygen deficits at low temperatures (MacMillan & Sinclair 2011). Prolonged exposure in this state leads to neuronal damage and eventually death, but shorter periods are reversible and carry no obvious short-term effects. The assay is therefore based upon eliciting a short-term coma in test subjects and then measuring the time taken to recover a given level of function once returned to a standard temperature above the CTM. The time frame for the initiation of a chill coma and the subsequent recovery varies among individuals, populations and species (Larsen & Lee 1994; Anderson *et al.* 2005; Arthur *et al.* 2008; Fischer *et al.* 2010), as well as according to the temperatures involved. We used a technique guided by the procedure of Davis *et al.* (2014) (see the following method) to increase the comparability of our results to previous studies.

Chill-coma assays

All F1 subjects ($n = 137$) experienced one chill-coma trial on the second day post-emergence and before they were released into the mating cage. F2 subjects ($n = 167$) experienced three trials over consecutive days, also starting on the second day post-emergence. We standardized the time of initiation of trials to mid-morning, mostly 10.00 hours but no later than 11.00 hours. For the trials, each test subject was transferred into a clean specimen jar (40-mm diameter × 60-mm height, volume 50 mL) and then transferred to a cool room at 3.0 ± 1.0 °C for 3 h. After 2 h, each jar was gently tapped to remove any butterflies clinging to the lid or sides, and to ensure that all individuals were lying flat at the bottom of the jar. At 3 h, the subjects were relocated to a laboratory bench at a temperature of 22.0 ± 1.0 °C. Recovery time was then measured, to the nearest second, as the time taken for each individual to right itself within the cup and remain standing.

In pilot observations, we noted that individuals may occasionally recover from chill coma yet not exhibit any outward physical signs of recovery. We therefore used a protocol of gently tapping each jar with a pen once every 2 min to stimulate test subjects to move (if possible). A total of three butterflies did not recover after 40 min. These individuals were subsequently found to have expired and were removed from the study.

Statistical analysis

The primary dependent variable – chill-coma recovery – approximates the incidence of a rare event at the timescales measured and has no negative values. Hence, this variable is likely to be based on an underlying Poisson probability function. Indeed, the raw data were non-normally distributed (Kolmogorov–Smirnov goodness of fit: $d = 0.179$, $P < 0.01$). We therefore used generalized linear models with a Poisson distribution for analyses because this distribution reduced the residual deviance of our models and is regularly used for time data with no negative values. Repeatability of the chill-coma assay across three consecutive trials on the F2s was first estimated using Pearson's product–moment correlations to account for differences in mean recovery time between trials. These correlation-based estimates of repeatability between individual

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trials were replicated using generalized linear mixed model (GLMM)-based repeatability (intra-class correlation coefficient) calculated on a latent scale with a Poisson distribution using recovery times from all three trials and individual as a random factor (Nakagawa & Schielzeth 2010). Here, a GLMM was used in the R package lme4 (Bates *et al*. 2015). An order effect (i.e. any consistent population-wise change in recovery time with advancing trials) was also tested using this GLMM, which also included sex as a fixed effect.

We estimated narrow-sense heritability using a Markov chain Monte Carlo GLMM (MCMCglmm) using data from both the F1 and F2 generations to estimate additive genetic variance as a random effect due to pedigree (i.e. genetic effects), along with the fixed effects of source population, sex, generation and body size (wing length). Here, we use the MCMCglmm package in R (Hadfield 2010) to conduct an animal model-based analysis (Wilson *et al*. 2010). This approach generates a design matrix based on the pedigree relationships among all individuals, including F0 grandparents (as field sampled and therefore with unknown chill-coma recovery time), F1 parents and their F2 offspring. Given that there was a pronounced order effect for chill-coma recovery across the three repeat trials conducted on F2s (Results section), we only included data from their first trial in this analysis. Random variance components were derived to estimate narrow-sense heritability ($h^2 \pm 1$ s.e.) on a latent scale because we specified a Poisson distribution for the MCMCglmm (Nakagawa & Schielzeth 2010). We also used the MCMCglmm approach to estimate genetic variances and heritability for the change in chill-coma recovery time across repeat trials, as experienced by the F2s. We used the change from trial 1 to trial 2 as the dependent variable in this analysis; source population, sex and body size were also included as fixed effects. Heritability was also calculated without fixed effects to make sure these factors were not overinflating the significance of our result. We expected some increase in h^2 with the inclusion of fixed effects. All MCMCglmm models were run for 300 000 iterations with a burn-in period of 30 000 and a thinning interval of 50 iterations.

RESULTS

Chill-coma recovery (time to recover from 3 h at 3.0 °C) ranged among individuals in the F1 generation from 60 to 2400 s and among F2 individuals (first trial) from 61 to 2350 s (Table 1).

Within-subjects variance: repeatability and trial order effects

Recovery time was positively correlated across all three trials for each F2 subject (trials 1 and 2: $r = 0.382$, $n = 157$, $P < 0.001$; trials 1 and 3: $r = 0.442$, $n = 153$, $P < 0.001$; trials 2 and 3: $r = 0.366$, $n = 153$, $P < 0.001$; Fig. 1), thus indicating repeatability at the level of individual phenotype by using the rank ordering of an individual's recovery time across trials. For all three pairwise comparisons, r was approximately 0.4, and repeatability calculated on a latent scale using a GLMM was similar ($r = 0.405$). The GLMM also indicated no effect of sex (Likelihood ratio test, $F_{1, 145} = 0.15$, $P = 0.91$) but a significant effect of trial order (Likelihood ratio test, $F_{2, 290} = 10.29$, $P < 0.01$). The order effect was such that individuals recovered faster on average in later trials, which proceeded irrespective of sex (Fig. 2). Figure 2 shows that trial 1 was significantly different to trials 2 and 3, but there was no significant difference between trials 2 and 3.

Table 1. Summary statistics for chill-coma recovery time in *Eurema smilax*

				Chill-coma recovery time (s)		
Generation	Population	Sex	*n*	Mean	95% CI	Range
F1	Narrabri	Female	37	457.8	390.9–524.7	150–1215
		Male	54	480.1	389.6–570.6	60–1430
	Quirindi	Female	24	535.5	329.5–741.4	60–2400
		Male	20	590.8	374.0–807.6	300–2400
	F1 subtotal		135	500.2	439.4–561.0	60–2400
F2	Narrabri	Female	53	470.2	381.0–559.4	61–2350
		Male	47	464.9	398.3–531.4	90–1340
	Quirindi	Female	7	608.4	213.0–1003.9	350–1568
		Male	9	535.3	325.8–744.9	255–1215
	Hybrid	Female	20	539.0	420.8–657.3	255–1215
		Male	24	509.8	434.1–585.5	270–1140
	F2 subtotal		160	492.9	450.6–535.2	61–2350
Total			295	496.2	460.4–532.1	60–2400

Data are summarized here using each F2 individual's first trial only. Samples exclude $n = 2$ (F1) and $n = 7$ (F2) individuals whose sex was unknown.

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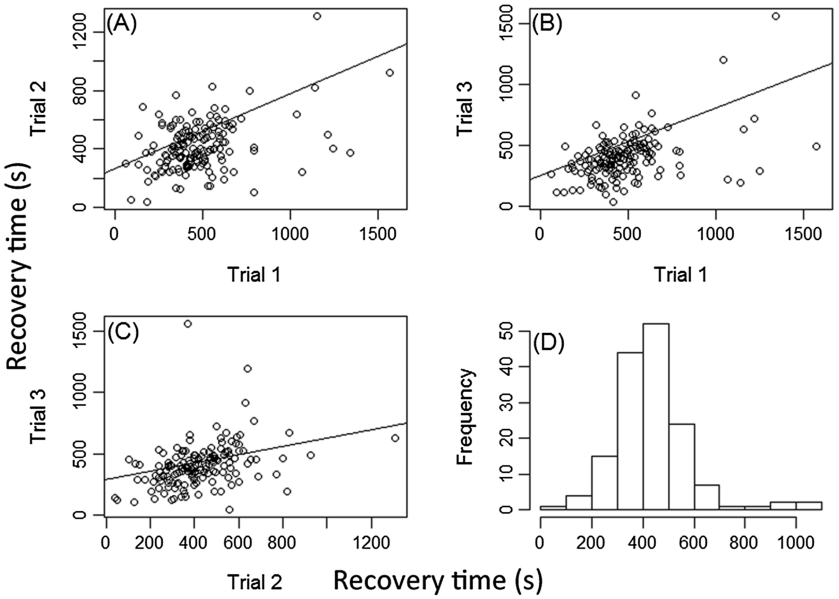

Fig. 1. Relationships between individual chill-coma recovery time among repeat trials for F2 individuals. Scatterplots indicate recovery time for (A) trials 1 and 2, (B) trials 1 and 3 and (C) trials 2 and 3. Panel D indicates the frequency distribution of recovery time (as averaged for each of the $n = 153$ individuals that participated in all three trials). Reduced major axis lines of best fit are indicated for panels A–C, and sample sizes are reported in the text.

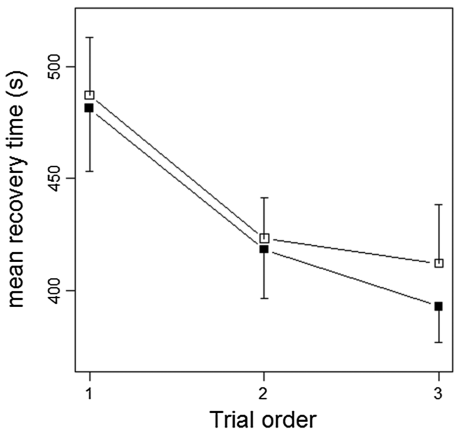

Fig. 2. Cold hardening effect across repeat trials. Mean recovery time for F2 individuals across each of three sequential chill-coma trials ($n = 147$; excluding individuals that did not complete all 3 trial or of unknown sex). Mean recovery times are indicated with s.e. bars. Closed squares = females; open squares = males. The mean of trial 1 is significantly different from 2 and 3, but trial 2 and 3 are not significantly different. There was no significant difference between the sexes.

Between-subjects variance: body size and genetic effects

A generalized linear mixed 'animal model' estimated a significant heritability value on a latent scale for chill-coma recovery of $h^2 = 0.414 \pm 0.100$ (Table 2). The fixed effects of sex, generation, source population and body size proved non-significant in this model (Table 2). Given the presence of a significant order effect (Fig. 2), we conducted a model using the change in chill-coma recovery from trials 1 to 2 as the dependent variable for phenotypic plasticity. This model proved non-significant, indicating that the cold hardening response itself lacks a genetic basis and is not related to sex or body size (Table 2).

DISCUSSION

Current climatic projections anticipate an increase in ambient temperature that may impact ectothermic species (Angert *et al.* 2011; Kingsolver *et al.* 2011). Some species have already started expanding their ranges into newly favourable areas (Ammunet *et al.* 2012; Pateman *et al.* 2012), and many other species are expected to be displaced from contemporary habitats because of intolerable thermal conditions (Wilson *et al.* 2005; Deutsch *et al.* 2008). Predicting changes in species distribution under climate change relies on the assumption that thermal tolerance was already limiting a species distribution. A large number of factors can regulate a species distribution, but quantifying thermal tolerance is a proactive first step towards predicting the vulnerability of a species to climate change. These changes in species distribution will have long-term impacts on the species composition of ecosystems and their ecology. Our effort to gain such data for *E. smilax* was motivated by a desire to assess the generality of paradigms gained in the extensively studied *Drosophila*

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Table 2. Summary of animal models (Markov chain Monte Carlo estimation method) for chill-coma recovery time and cold hardening in *Eurema smilax*

	Recovery time	Cold hardening
Fixed effects	Posterior mean (95% C.I.)	Posterior mean (95% C.I.)
Intercept	5.969 (4.871–7.013)	328.40 (−360.06–1001.30)
Generation	−0.050 (−0.195–0.094)	—
Sex	0.040 (−0.088–0.165)	−13.44 (−85.45–61.46)
Body size	0.014 (−0.050–0.076)	−13.86 (−54.48–26.36)
Population (Narrabri)	−0.149 (−0.419–0.158)	−58.74 (−205.28–92.04)
Population (Quirindi)	−0.030 (−0.332–0.295)	28.35 (−217.44–230.71)
Random effects		
Additive G	0.133 (0.061–0.216)	22167 (0.26–67385)
Residual	0.182 (0.123–0.237)	34821 (67.59–51521)
$h^2 \pm 1.0$ s.e.	**0.414 ± 0.100**	0.358 ± 0.224
$h^2 \pm 1.0$ s.e. without fixed effects	**0.397 ± 0.094**	0.325 ± 0.193

Significant ($P < 0.05$) effects are highlighted in bold type. Additive genetic variance ('Additive G') was estimated using the animal model, which accounts for the full genetic relationship matrix across the F0–F2 generations ($n = 52$ F0s; $n = 137$ F1s [minus 2 of unknown sex]; $n = 167$ F2s [minus 7 of unknown sex]; total $n = 347$). The cold hardening response was measured by the change in recovery time from trials 1 to 2 in *E. smilax*. Generation effects are omitted in the cold hardening analysis because only F2s experienced repeat trials (hence precluding this calculation in the F1 generation). For the fixed effect of source population, there are three categories: Narrabri, Quirindi and mixed; in this case, the mixed category acts as the intercept, and neither Narrabri nor Quirindi was significantly different from the intercept or each other. We reran each model with the fixed effects removed, and these heritability estimates are given at the bottom of the table. Adding fixed effects to models will always inflate h^2, but in this case, the difference seems negligible.

model system. As predicted, given the broad distribution of *E. smilax*, our investigation revealed significant additive genetic variance for intrinsic cold tolerance. This implies significant adaptive potential for this trait. We also revealed that individual cold tolerance increases with prior exposure to sub-lethal temperatures (seen as a reduction in recovery time over consecutive trials; Fig. 2), akin to the plastic 'cold hardening' response reported for other invertebrates (Hoffmann *et al.* 2003b; Fischer *et al.* 2010; Mitchell *et al.* 2011). The magnitude of this response was not heritable (Table 2) and hence is itself unlikely to readily evolve. We explore these findings in turn in the following.

Genetic variation

Through estimating narrow-sense heritability, we estimated the proportion of variation in cold tolerance, which is accounted for by additive genetic variation. In an earlier study, Davis *et al.* (2014) measured broad-sense heritability (H^2) in *E. smilax*, *E. hecabe* and *E. laeta*, showing that *E. smilax* (the species with the widest distribution) recovered fastest from a standard chill-coma assay and also exhibited the greatest broad-sense heritability for this trait ($H^2 = 0.327$; $n = 79$). This result is directly comparable with ours because both studies sourced individuals from a population in the Narrabri region of NSW, Australia. Our data suggest that most of the previously reported genetic variance for cold tolerance in this population (Davis *et al.* 2014) is additive in nature and would therefore respond to selection.

Our results are broadly consistent with the range of heritability values estimated for cold tolerance in other invertebrates. Long-term selection experiments in *Drosophila melanogaster* have furnished realized heritability (h^2) for this trait ranging from 0.14 in a population from Belgrade in south-east Europe (Tucić 1979) to 0.46 in populations sourced from north-eastern Australia (Anderson *et al.* 2005). In the tropical African butterfly *Bicyclus anynana*, realized heritability averaged 0.098 ± 0.03 across four selected outbred lines, with lower values reported for lines subject to experimental inbreeding (Dierks *et al.* 2012a). Our estimate of cold tolerance heritability in *E. smilax* ($h^2 = 0.414 \pm 0.100$) cannot be compared statistically with other estimates. Comparing heritability estimates can be difficult because of differences in methodology (e.g. the rate of temperature change during the recovery stage) could affect the variance of results in different experiments, which confounds the direct comparison of heritability estimates among studies (see Chown *et al.* 2009 for a full discussion). Although our data are unlikely to represent the entirety of Australian *E. smilax*, they do provide a valuable reference point for future work in other populations, and for comparison between widespread and tropically restricted Australian congenerics.

The distance between our sampling sites created the possibility that genetic differentiation between our two sampling locations could affect our results. However, the results presented no evidence for population-level differences in chill-coma recovery time (Tables 1 and 2).

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The sampling sites represent two locations within a continuous range of suitable habitat, and given the high mobility of the species (Smithers 1983; Braby 2000), this implies that we sampled from a single genetic population.

Phenotypic plasticity

The hardening response described here (Fig. 2) exemplifies the plastic responses to temperatures below their CTM reported in some insects, including *D. melanogaster* (Chen & Walker 1993; Mitchell *et al.* 2011), flesh flies (*Sarcophaga crassipalpis*) (Chen & Denlinger 1992) and butterflies (*Danaus plexippus*, Larsen & Lee 1994, and *B. anynana*, Fischer *et al.* 2010; Franke *et al.* 2012). Plasticity in cold tolerance may potentially involve physiological mechanisms such as membrane fluidity (Hazel 1995; Ohtsu *et al.* 1998; Overgaard *et al.* 2005) and mitochondrial capacity (Pörtner 2002). As a result, plasticity is advantageous because of a delayed investment of resources (Hoffmann *et al.* 2003b; Ayrinhac *et al.* 2004; MacMillan & Sinclair 2011). Phenotypic plasticity is generally expected to be adaptive for widely distributed species that need to cope with changing conditions through space and time (Gienapp *et al.* 2008). In this case, the broad distribution of *E. smilax* across Australia could have resulted in selection for high plasticity in cold tolerance.

Individual repeatability

In order to offer a truly representative measure of the intrinsic capacity for cold tolerance (given that such an intrinsic component exists), an assay must produce results that are repeatable at the individual level (Anderson *et al.* 2005; Hazell *et al.* 2008). Repeat trials conducted on second-generation individuals indicated that such repeatability is indeed present (Fig. 1); that is, the rank ordering and scaling of individual values relative to each other were consistent across trials (even given sample-wide variation in absolute recovery time between trials). Using a GLMM with individual as a random factor, we found repeatability (intra-class correlation coefficient) of 0.405 for chill-coma recovery time. This result indicates a small majority of the variance in chill-comma recovery is within individuals rather than between. However, this within-individual variance could be lower without the hardening effect that reduces recovery time across trials that will increase variance within individuals. Therefore, this estimate of repeatability could be higher without the hardening effect, which is a result of individuals' acclimatizing to cold exposure. Our protocol included the light tapping of specimen jars every 2 min during the recovery period, an approach that has elsewhere been questioned (Hazell *et al.* 2008). However, our primary interest was to assay the precise point when individuals recovered neuro-muscular function (Hazel 1995; MacMillan & Sinclair 2011), and pilot trials indicated high potential for some individuals to reach this point yet not reveal it behaviourally. We therefore view the inclusion of a stimulus as necessary for reducing the error variation, which might otherwise arise because of motivation, a point that could be usefully explored via more detailed future work.

Conclusion

Research on butterflies has already observed distributional shifts in response to climate change (Wilson *et al.* 2005; Pateman *et al.* 2012) that is consistent with patterns seen in other species (Sunday *et al.* 2012). An alternative biological response, as emphasized by the work of Hoffmann and colleagues (Hoffmann *et al.* 2003b; Anderson *et al.* 2005; Kellermann *et al.* 2009; Hoffmann 2010), is that marginal populations may instead evolve to track the changing thermal regimes. Very few studies have documented microevolutionary responses in wild populations (Gienapp *et al.* 2008; Hoffmann & Sgro 2011), although substantial evidence for evolutionary potential has been gained through selection experiments (Tucić 1979; Hoffmann *et al.* 2003a; Dierks *et al.* 2012a). The high heritability and phenotypic plasticity for cold tolerance in the studied population of *E. smilax* suggest this species is an excellent candidate for examining microevolution across a broad climatic distribution (ranging from the cool temperate to tropical and arid regions of mainland Australia) over a range much wider than our sampling region. Both genetic and epigenetic variations could explain population-level variation in cold tolerance that is correlated with local climatic conditions. Future research could also expand upon comparisons between *E. smilax* and its more geographically restricted Australian congenerics (Davis *et al.* 2014). Such work can assist in identifying species of primary conservation concern under future climate scenarios.

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