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Low genetic variation in cold tolerance linked to species distributions in butterflies

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Abstract Species with restricted distributions make up the vast majority of biodiversity. Recent evidence from *Drosophila* suggests that species with restricted distributions may simply lack genetic variation in key traits, limiting their ability to adapt to conditions beyond their current range. Specifically, tropical species of *Drosophila* have been shown to have low means and low genetic variation for cold tolerance and desiccation tolerance. It has therefore been predicted that these species will be limited in their response to future climatic changes. However whether these results extend beyond *Drosophila* is not known. We assess levels of quantitative genetic variation for cold tolerance and body size in three species of butterfly from the genus *Eurema* that can be classified as tropically restricted (*E. laeta*), tropical/subtropical (*E. hecabe*) and widespread (*E. smilax*) in their distribution. Compared to the more widely distributed species, we show that the tropically restricted *E. laeta* has significantly lower mean cold tolerance and lacks genetic variation for this trait. Thus, we empirically confirm in non-model organisms that low levels of genetic variation in a key ecological trait may play a role in limiting the distribution of tropically restricted species.

Keywords Heritability · Genetic variation · Adaptation · Cold tolerance · Butterfly

Introduction

Heritable variation is essential for any adaptive response to environmental change. It has been long assumed that most traits in wild animal and plant populations are heritable and

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are able to respond to natural selection. This is because it has been assumed that a balance between mutation generating new variants and stabilising selection decreasing genetic variation will maintain significant levels of heritable genetic variation. Hence, adaptation and evolution should not be limited by a lack of genetic variation, except on rare occasions (Blows and Hoffmann 2005). Recent data collected for the model system *Drosophila* challenges this view (Kellermann et al. 2006, 2009).

Specifically, tropical *Drosophila* species with narrow geographic distributions have very low levels of genetic variation in cold and desiccation tolerance. In contrast widely distributed *Drosophila* species have much higher levels of genetic variation in these same traits (Kellermann et al. 2009). Significant levels of genetic variation for body size were present in both tropical and widespread species, indicating that these differences were not due to low overall genetic diversity in the tropically restricted species. Rather, the fact that both cold (Kimura 2004; Parsons 1982; Gibert et al. 2001; Kellermann et al. 2012a, b) and desiccation (Parsons 1982; Van Herrewegge and David 1997; Kellermann et al. 2012a, b) tolerance have been linked to differences in species distributions, suggests that levels of genetic variation in key ecological traits are driving differences in physiological tolerances between tropical and widespread *Drosophila* species. In particular, Kellermann et al. (2009) suggest that their results support the hypothesis that specialisation to tropical environments in *Drosophila* species reflects fundamental genetic limits in ecologically important traits. That is, tropically restricted species may lack the genetic variation in key traits and thus be constrained in their ability to mount evolutionary responses to changing environmental conditions.

While the idea that genetic variation might limit evolution is not new (Bradshaw 1991) few studies have explicitly considered the extent to which low levels of genetic variation in ecologically important traits may indeed limit evolutionary responses (reviewed in Futuyma 2010; but see McGuigan et al. 2008). However such studies are central to understanding the factors that limit species from expanding beyond their current range (Buckley et al. 2012). The results of Kellermann et al. (2006, 2009) in *Drosophila* suggest that species with restricted distributions and populations at species borders may simply lack genetic variation in ecologically important traits, limiting their ability to adapt to conditions beyond their current range. If this is a general phenomenon, then these types of species and populations are likely to be fundamentally constrained in their evolutionary response to future climate change, and other threats, and will face high risks of extinction. Whether the findings in *Drosophila* apply more broadly to other tropically restricted species is presently unknown.

Here, we used full-sib families to assess levels of quantitative genetic variation for the ecologically important trait of cold tolerance, in three congeneric species of butterfly (genus *Eurema*) that differ in their level of climatic specialisation. Butterflies have been intensely studied, providing some of the first evidence for species range shifts and contractions in response to climate change. Most of these studies have focussed on temperature limitation of dispersal ability and life history traits (e.g. Klemme and Hanski 2009; Buckley et al. 2012). While cold tolerance has been associated with range limits in butterflies (Crozier 2003, 2004a, b), no studies have linked levels of adaptive genetic diversity in cold tolerance to range limits.

Eurema species are distributed throughout tropical, sub-tropical and temperate regions (Braby 2000). The three species chosen for this study exhibit highly contrasting Australian distributions (Braby 2000), which allows them to be classified as tropically restricted (*E. laeta*), tropical/subtropical (*E. hecabe*) and widespread (*E. smilax*). All three species utilise a range of host plants whose distribution greatly exceeds their extant geographic range,

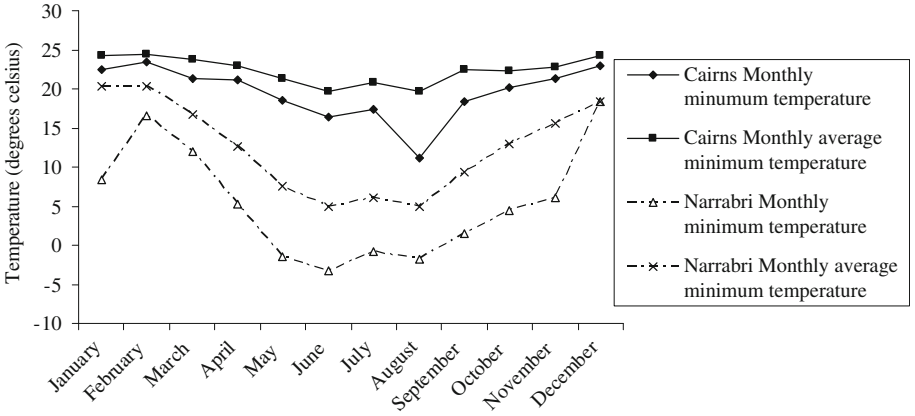

Fig. 1 Minimum temperature data for the two sampling locations. Data were collated from Cairns Aero (16.87°S, 145.75°E) and Narrabri Airport (30.32°S, 149.83°E). All temperatures are recorded in °C

thus we can a priori rule out the influence of host plant distribution (Braby 2000). Furthermore, there are clear differences in the minimum temperatures experienced by these species towards the southern parts of their distribution (Fig. 1) and the development of *E. laeta* and *E. hecabe*, but not *E. smilax*, seems to be limited by cooler temperatures (Jones 1992). This suggests that cold tolerance may play a role in the distributional limits of these species. If the results from *Drosophila* apply more broadly, we predict that the tropically restricted *E. laeta* will be most susceptible to cold stress, and will display significantly lower levels of genetic variation for cold tolerance than either of the two more widely distributed species. *E. hecabe*, with its intermediate distribution, should fall in between *E. laeta* and *E. smilax* for mean cold tolerance and levels of genetic variation for this trait.

Materials and methods

Field collections

Female *Eurema laeta* and *E. hecabe* were collected from Cairns (16.87°S′ 145.75°E) Queensland, Australia, in March 2010. *Eurema smilax* females were collected from Narrabri (30°33′S, 149°78′E), New South Wales, in April 2010. Since *Eurema* butterflies mate soon after emerging, most free-flying adult females have already mated (Kemp and Macedonia 2007). The field collected females were used to set up 16 families of *E. laeta* 81 families of *E. hecabe* and 12 families of *E. smilax*. The disparity in numbers of butterflies collected is due to the fact that the tropically restricted species are very abundant during the wetter months, while the widespread species is more of a vagrant, and is difficult to find in large numbers in one location.

Laboratory rearing

Laboratory populations of *Eurema* were reared following the procedures established by Kemp and Macedonia (2007) and Kemp and Rutowski (2007). Females were kept in separate plastic cups (diameter 90 mm, height 140 mm) containing cuttings of the host-

plant for oviposition. Host-plant species included *Chaemachrista nomane* var. *nomame* (*E. laeta*), *Neptunia gracilis* (*E. smilax*), and *Sesbania cannabina* (*E. hecabe*). All plants were grown in a greenhouse facility at Macquarie University (set at 25 ± 1 °C [day] and 20 ± 1 °C [night]). Females from all species were placed under full-spectrum 400 W metal-halide lighting for short periods in order to promote oviposition. Females that had produced 15–20 eggs were taken from the cups, placed in envelopes and stored at −20 ± 1 °C. The cups were placed aside to wait for eggs to hatch, which takes approximately 3 days.

After hatching, caterpillars were put in fresh cups, and kept at low densities—in groups of four to six—with host plant supplied ad libitum. Upon eclosion, family, gender and date of emergence were recorded. Each individual was marked with a unique identifier on the right hind wing using a permanent marker. The laboratory was maintained at an average temperature of 22.5 ± 1 °C. Larval survival under these rearing conditions for all three species was approximately 90 %, reflecting non-stressful rearing conditions.

Assessing cold tolerance and body size

Chill-coma recovery time is widely used to assess cold tolerance in insects (Ransberry et al. 2011). We assessed chill-coma recovery time and body size for each species. For *E. laeta* and *E. hecabe* experiments were performed using the F2 offspring of field caught females. For *E. smilax*, due to limitations on the number of rearings, experiments were performed on the F1 offspring of field-caught females. Chill-coma recovery time was measured as the time taken for butterflies to recover from a 3 h exposure to 4 °C. Butterflies subjected to a chill-coma will lie unresponsive on their side, and were scored as 'recovered' once they are able to right themselves. Body size was assessed by measuring the length (to the nearest 0.01 mm using digital calipers) of the right forewing from outer edge to the thoracic insertion point (Kemp 2008). Between 2 and 20 offspring were assessed for each trait from each family.

Statistical analyses

An analysis of covariance (ANCOVA) was first performed within each species to test for effects of sex and wing length (fixed factors) on mean cold tolerance. An additional model (Overall Model) was then used to test for differences in mean cold tolerance between species. Sex and wing length were excluded from this model, since they were shown to have non-significant effects on cold tolerance within each species. A second ANCOVA was performed to test for effects of sex on wing length. An additional model (Overall Model) was then used to test for differences in mean wing length between species. Post-hoc comparisons between species for cold tolerance and wing length were conducted using Tukey's multiple comparison tests, correcting for multiple comparisons.

Estimates of broad-sense heritability were obtained by performing one-way analyses of variance (ANOVA's) for each species, with family as a random factor. Since sex and body size were shown to have non-significant effects on cold tolerance as indicated by the ANCOVA's described above, they were excluded from the analysis for this trait. Sex did have a significant effect on body size in *E. hecabe*, so analyses for this trait were conducted on sexes separately. Significant between family effects indicate significant levels of genetic variance for each trait (Lynch and Walsh 1998). As the design was highly unbalanced, variance components were calculated via the restricted maximum likelihood method

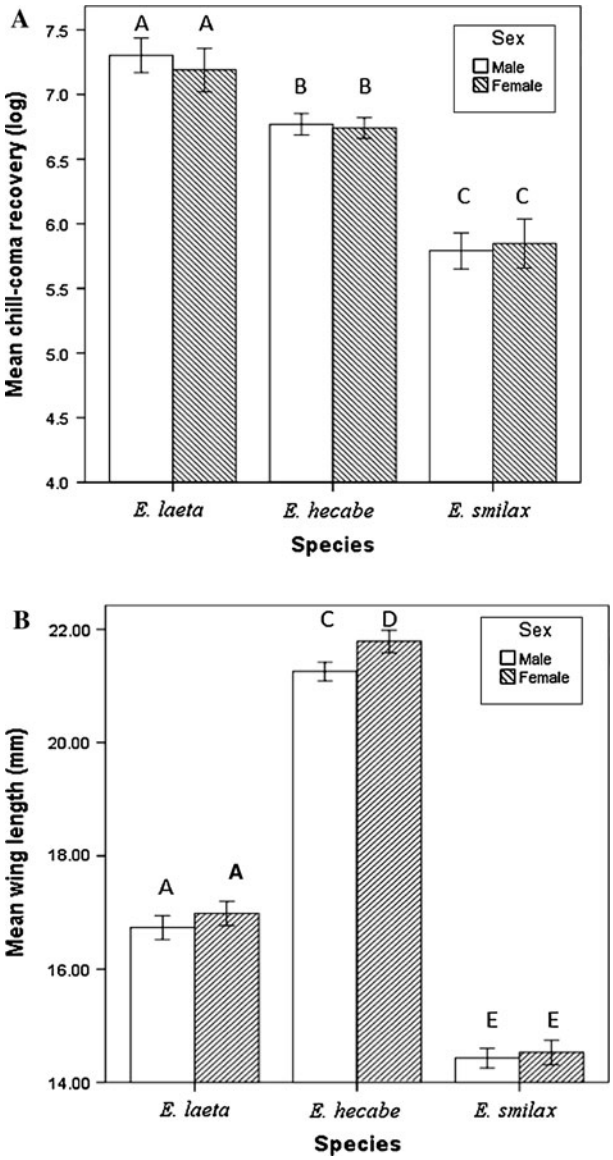

Fig. 2 Mean chill-coma recovery time (**a**) and mean body size (**b**) for the three species examined: *E. laeta* (tropical), *E. hecabe* (sub-tropical) and *E. smilax* (widespread). *Different letters* above *bars* indicate significant differences at $P < 0.05$ after correcting for multiple tests (Tukey's multiple comparison test)

(REML) (Kemp and Rutowski 2007; Lynch and Walsh 1998) and estimates of broad sense heritability (H^2) calculated as per Lynch and Walsh (1998).

Results

As predicted, *Eurema smilax* (widespread) was significantly more cold tolerant than *E. hecabe* (sub-tropical), and both were significantly more tolerant than *E. laeta* (tropical) (Fig. 2a; Table 1a). No such patterns were evident for body size (Fig. 2b; Table 1b), and size and sex had no effect on cold tolerance. *Eurema smilax* (widespread) and *E. hecabe*

Table 1 Analyses of covariance testing for A) effects of wing length and sex on mean cold tolerance and B) effects of sex on mean wing length, for each species separately

Species	*N*	Factor	*df*	Mean square	*F*	*P*
A. Cold tolerance						
E. laeta	152	Wing length	1	1.421	3.396	0.067
		Sex	1	0.450	1.075	0.302
E. hecabe	284	Wing length	1	0.201	0.477	0.490
		Sex	1	0.000	0.000	0.986
E. smilax	86	Wing length	1	11.480	1.322	0.254
		Sex	1	0.354	0.279	0.598
Overall model	746	Species	2	57.460	137.938	<0.005
B. Wing length						
E. laeta	Males-91	Sex	1	2.299	2.534	0.114
	Females-62					
E. hecabe	Males-146	Sex	1	20.534	16.961	<0.005
	Females-138					
E. smilax	Males-52	Sex	1	0.418	0.518	0.473
	Females-34					
Overall model	523	Species	2	2,888.300	2,740.066	<0.005

Overall model tests for differences between species for each trait. N = number of individuals

(sub-tropical) displayed significant levels of quantitative genetic variation for cold tolerance as indicated by the significant Family terms and broad-sense heritability estimates (Table 2a; Fig. 3), but *E. laeta* (tropical) did not (Fig. 3; Table 2a). All three species displayed significant levels of genetic variation for body size (Fig. 3; Table 2b) that was not related to their geographic distribution.

Discussion

Temperature is a key environmental factor influencing species' ranges in most organisms, particularly ectotherms. In particular, cold tolerance has been linked to the distribution of *Drosophila* and other insects (Chown et al. 2002). Tropically restricted species have lower physiological tolerances, and are therefore more sensitive, to cold stress than widespread, temperate species (Addo-Bediako et al. 2000). Recent work in *Drosophila* (Kellermann et al. 2009) suggests that low levels of genetic variation in cold tolerance, may drive these differences in physiological tolerances between tropical and widespread species. Studies testing these patterns in other taxa are needed to better understand the factors that drive species abilities to adapt to changing environmental conditions (Buckley et al. 2012).

While butterflies are seen as sentinels of current and future climate change (e.g. Hellmann 2002; Walther et al. 2002; Crozier 2003; Parmesan 2006; Hellmann et al. 2008; Thomas 2010), most studies have focussed on species from the temperate northern hemisphere, and none have explicitly assessed levels of genetic variation in cold tolerance and linked this variation to species distributional limits.

Using three congeneric species of butterfly (genus *Eurema*) that differ in their level of climatic specialisation, we set out to determine whether the pattern of reduced genetic

Table 2 Analyses of variance testing for significant differences between families for A) cold tolerance, and B) wing length

Species		*N*	Range (N per family)		*df*	Mean square	F	*P*	$V_{(family)}$	H^2
A. cold tolerance										
E. laeta		115	4–20	Family	15	0.620	1.380	0.172	0.031	0.131
				Error	99	0.449				
E. hecabe		494	2–9	Family	80	0.774	2.060	<0.005	0.065	0.294*
				Error	413	0.376				
E. smilax		79	4–14	Family	11	0.507	2.252	0.021	0.044	0.327*
				Error	67	0.225				
B. Wing length										
E. laeta	M	100	2–14	Family	28	2.028	2.947	<0.005	0.365	0.696*
				Error	71	0.688				
	F	61	2–7	Family	19	1.584	3.345	0.001	0.377	0.888*
				Error	41	0.474				
E. hecabe	M	140	2–5	Family	46	1.741	2.534	<0.005	0.358	0.685*
				Error	93	0.687				
	F	121	2–4	Family	43	1.995	1.889	0.006	0.310	0.451*
				Error	87	1.056				
E. smilax	M	91	4–14	Family	12	1.425	2.559	0.007	0.135	0.386*
				Error	78	0.557				
	F	74	4–12	Family	11	2.125	3.826	<0.005	0.278	0.665*
				Error	62	0.556				

Broad sense heritability estimates (H^2) are also presented for each trait. *M* males, *F* females, *N* number of individuals

* $P < 0.05$

variation for cold tolerance in tropically restricted species observed so far only in *Drosophila* holds true across other taxa of invertebrates.

As predicted, we found low levels of genetic variation for cold tolerance, but not wing size, in the most tropically restricted and cold sensitive species (*E. laeta*). Importantly, the development of *E. laeta* and *E. hecabe,* but not *E. smilax,* is sensitive to low temperature, with poor survival below 22 °C (Jones 1992). Combined with the results of the current study, it would seem that the capacity for *E. laeta* to expand its geographic range by evolving higher levels of cold tolerance in response to the colder conditions found beyond the wet tropics is limited by low levels of quantitative genetic diversity for this trait. The observation that this pattern extends beyond the *Drosophila* group indicates that low genetic variation in key ecological traits important for surviving beyond their tropical distribution may well provide a general explanation for distributional limits in tropically restricted species. Whether this pattern extends to other climatic stress traits such as heat tolerance, which will be important for understanding responses to future climate changes, is not clear. However recent data suggests responses to heat may be constrained across many species (Kellermann et al. 2012a, b; Kelly et al. 2012).

It is not clear what mechanisms might be driving these results. Hypotheses involving asymmetrical gene flow that might account for evolutionary stasis do not predict that low

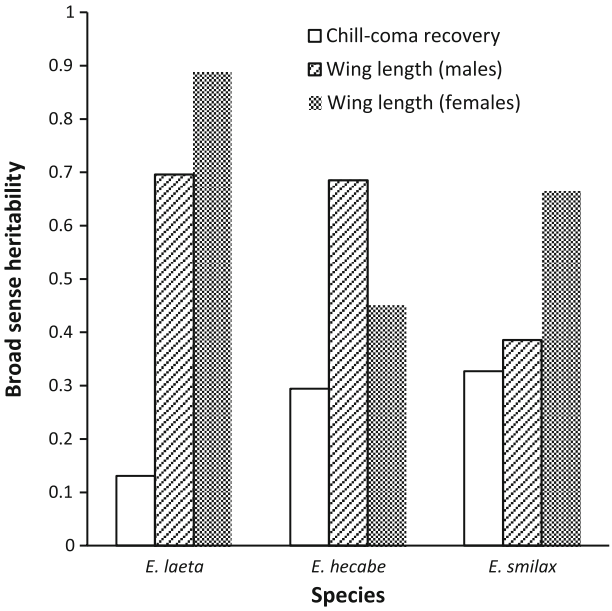

Fig. 3 Broad sense heritability estimates for chill-coma recovery time and body size for the three species examined: *E. laeta* (tropical), *E. hecabe* (sub-tropical) and *E. smilax* (widespread)

levels of genetic variation will limit evolutionary responses (Blows and Hoffmann 2005). Significant levels of genetic diversity for body size in all three species rules out loss of genetic diversity due to small population sizes. Similarly, if directional selection was responsible for the low genetic variation in *E. laeta* by driving alleles to fixation, we would expect to see low genetic variation coupled with high (directionally selected) mean cold tolerance (Blows and Hoffmann 2005). This is not the case. Other hypotheses invoking loss of genetic diversity via DNA decay or the possibility that tropical species may be evolutionary derived, or that the more widespread species are derived following gene duplication events that increase their resistance and genetic diversity remain to be tested (Kellermann et al. 2009). Trade-offs could also be involved if they produce strong directional selection for sensitivity to cold in tropically restricted species (Blows and Hoffmann 2005).

We used full-sib families to estimate quantitative genetic variation for cold tolerance and body size in this study. Our estimates of genetic variation and broad-sense heritability therefore set an upper limit for genetic variation within these traits, since they may include contributions from non-additive sources of genetic variation. However our estimates of broad-sense heritability for cold tolerance across the three species are similar in magnitude to the narrow-sense heritability estimates reported for the same trait across *Drosophila* species (Kellermann et al. 2009), suggesting perhaps that our estimates are not significantly inflated by non-additive effects.

It is also possible that our results for *E. laeta* reflect limited power to detect significant levels of genetic variation due to small samples sizes. However we feel that this is unlikely for two reasons. Firstly, *E. smilax*, with the smallest number of families ($N = 12$) still displayed significant levels of genetic variation for cold tolerance, and secondly, all three species displayed similarly high levels of genetic variation for body size. Finally, it is also possible that the estimates of genetic variation obtained in this study from a single population of each species may not represent patterns of genetic variation across the entire

range of each species. Studies across multiple populations of rainforest restricted *Drosophila* species (Kellermann et al. 2006), however, confirm that single population estimates can accurately capture species level differences in genetic variation.

In conclusion, our results confirm the results reported so far only in *Drosophila*: low levels of quantitative genetic variation in ecologically important traits may indeed play a major role in driving the limited distributions of tropically restricted species. The challenge remains to uncover the mechanisms behind these patterns.

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