Disparate patterns of thermal adaptation between life stages in temperate vs. tropical populations of *Drosophila melanogaster*

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Abstract

Ectotherms may be threatened by increasing global temperatures, particularly if thermal adaptation is slower than climate change, as recent work has suggested. But estimates of evolutionary rates depend on the strength of natural selection, which may vary across life stages. Thus, depending on the life stage examined, comparisons of thermal tolerance among populations and species may yield inaccurate estimates of the rate at which thermal tolerance can evolve. Here we compared acute thermal tolerance in two life stages, adults and embryos, among populations of *Drosophila melanogaster* that span a broad range of thermal habitats across temperate sites in eastern North America and tropical sites around the globe. We report no variation in adult thermal tolerance among populations across this broad geographic range. In contrast, embryonic thermal tolerance was significantly higher in individuals from tropical sites compared to temperate sites. Embryonic thermal tolerance was similar among temperate populations, but among tropical populations embryonic thermal tolerance was positively correlated with maximum habitat temperature. We further report that embryos live closer to their upper thermal limits than adults—i.e., thermal safety margins are smaller for embryos than adults. F1 hybrid embryos from crosses between temperate and tropical populations had embryonic thermal tolerance that matched that of tropical embryos, suggesting phenotypic dominance of heat-tolerant alleles. Together our findings demonstrate that thermal tolerance readily evolves in the earliest and most thermally sensitive life stage and suggest that thermal selection may be more limited in the adult life stage. Disparate patterns of embryonic thermal tolerance between temperate and tropical populations may be the result of regional differences in seasonality.

Introduction

Extreme temperatures, which may be encountered at the edge of a species' geographic range (Hilbish et al., 2010) or episodically during the hottest or coldest days of the year (Hoffmann, 2010; Dowd et al., 2015; Buckley and Huey, 2016), can cause populations to experience mortality (Helmuth et al., 2002; Denny et al., 2006) and ultimately lead to thermal adaptation (Lenski and Bennett, 1993; Mongold et al., 1999; Hangartner and Hoffmann, 2015). However, recent work suggests that thermal adaptation of upper thermal limits may be evolutionarily constrained (Hoffmann et al., 2013; Schou et al., 2014; Hangartner and Hoffmann, 2015; Kristensen et al., 2015; van Heerwaarden et al., 2016), such that the evolution of increased heat tolerance may be a relatively slow process that takes many millions of years to achieve (Kellermann et al., 2012). If this is the case, global climate change, which has led to rapid increases in mean temperatures and the frequency of extreme thermal events (Katz and Brown, 1992; Meehl et al., 2000; Cai et al., 2014), may cause shifts in geographic distributions (Rank and Dahlhoff, 2002; Burrows et al., 2011; Thomas et al., 2012; Sunday et al., 2012) as populations may not be able to adapt fast enough to persist in hotter environments.

But evolutionary rates depend on several factors that may not be taken into account in estimates of thermal adaptive potential. In particular, adaptive rates depend on the strength of natural selection (Bennett et al., 1992; Rudolph et al., 2010), and studies that focus on thermal tolerance of mobile organisms or life stages may over estimate the degree to which these organisms encounter thermal selection in nature. In other words, thermal safety margins—i.e., the difference between upper thermal limits and maximum habitat temperature—may be larger than predicted because thermal environmental heterogeneity allows mobile organisms to avoid thermal extremes via behavioral thermoregulation (Dillon et al., 2009; Gunderson and Leal, 2012). To date, there have been relatively few studies that examine thermal adaptation in immobile organisms or life stages, particularly in the terrestrial realm (Angilletta et al., 2013; MacLean et al., 2016), and immobile organisms may represent ideal study systems to investigate the evolutionary potential of thermal tolerance. In support of this conjecture, broad scale patterns of thermal tolerance are more tightly correlated with habitat temperatures in marine systems than in terrestrial systems (Sunday et al., 2011), perhaps due to the more limited range of thermal microhabitats in the marine realm (Denny et al., 2011) that makes behavioral thermoregulation a less effective buffering mechanism.

Here we sought to compare adult and embryonic heat tolerance among populations of fruit flies, *Drosophila melanogaster*, from a broad range of thermal habitats across the world to ascertain the degree to which thermal selection has shaped the evolution of thermal tolerance across immobile vs. mobile life stages. Adult thermal tolerance has been extensively studied in natural populations of *D. melanogaster* —particularly in eastern Australia (Hoffmann et al., 2002; Sgro et al., 2010; Buckley and Huey, 2016)—but to a large extent, the thermal physiology of the early embryonic life stage of *D. melanogaster* has not been characterized in natural populations (Kristensen et al., 2015). Studies of lab-bred *D. melanogaster* have shown that early embryos (0 – 2 hours post-fertilization) are more thermally sensitive than later stages (Walter et

al., 1990), perhaps due to the reduced heat-shock response in early embryos (Graziosi et al., 1980; Welte et al., 1993). Thus, we compared heat tolerance of adults and early stage embryos to determine whether or not differences in thermal sensitivity, as well as mobility, lead to different patterns of thermal adaptation across life stages. The thermal environment of *D. melanogaster* can change rapidly (+18°C h^{-1}) and reach extreme values (> 40°C) (Feder et al., 1997). Therefore, we designed our thermal stress experiments to mimic sudden (acute) changes in temperature that are characteristic of the variable thermal environments that flies experience in nature (Terblanche et al., 2011). We report higher embryonic thermal tolerance in tropical (hotter) vs. temperate (cooler) populations but no difference in adult thermal tolerance, and thus we demonstrate that thermal selection likely varies across life stages. Moreover, our data suggest that there is significant adaptive potential for upper thermal limits in the earliest and most thermally sensitive life stage.

Results

Thermal tolerance and thermal safety margins across life stages

We found no difference in adult thermal tolerance among all sites (Figs. 1B and 1C), with an overall mean CT_{max} (± 95% C.I.) of 38.8 ± 0.45°C. Embryonic thermal tolerance (LT_{50}) did not differ among the three temperate sites but was significantly higher in tropical vs. temperate embryos (Figs. 1D and 1E). Overall, tropical embryos were more heat tolerant; the average LT_{50} was approximately 1°C higher in tropical embryos (35.8 ± 0.45°C) than in temperate embryos (34.88 ± 0.18°C). There was no significant relationship between adult CT_{max} and embryo LT_{50} (Fig. S1).

Thermal safety margins—i.e., the difference between thermal tolerance (CT_{max} or LT_{50}) and maximum habitat temperature (T_{max})—were consistently smaller for embryos than adults and smallest in the tropics. This pattern was consistent regardless of whether thermal safety margins were compared across latitude (Fig. 2A) or by region (temperate vs. tropical) (Fig. 2B).

Climate variability and thermal tolerance

While there is a consistent difference between our temperate and tropical sites in mean annual temperature, VT = 5.4°C, IN = 10.8°C, NC = 15.4°C, and all tropical = 26.1 ± 1.4°C (mean ± 95% C.I.), a principal components analysis of 19 Bioclim variables indicated that the greatest difference in climate among the collection locations was in temperature seasonality—defined as the yearly standard deviation in temperature. PC1 explained 95.6% of the variation in climate data among the collection sites, and this pattern largely characterized the differences in climate between temperate vs. tropical sites (Fig. S2). Temperature seasonality was the variable with the top component loading of PC1, explaining 98% of the variation in PC1. There was a significant negative relationship between PC1 and embryonic heat tolerance (Multiple least-squares linear regression with PC1 and PC2 as covariates, $F_{1,4} = 8.1474$, $P = 0.0462$), but this pattern was driven by the bimodal distribution of LT_{50}s between temperate vs. tropical sites and was not consistent with temperature seasonality within each geographic region (Fig. S3). PC2 explained

4% of the variation in climate data among collection sites, mostly characterizing the differences in climate among tropical sites (Fig. S2). Annual precipitation, precipitation of the wettest quarter, and precipitation of the wettest month were the top three component loadings of PC2, explaining 49%, 40%, and 8% of variation in PC2, respectively. But there was no significant relationship between PC2 and embryonic heat tolerance (Least-squares linear regression, $F_{1,4}$ = 0.367, P = 0.577).

Maximum temperature of the warmest month (i.e., maximum habitat temperature or T_{max}) spanned a range of 8.4°C among all sites, from 25.7°C in Vermont, USA (VT) to 34.1°C in Chiapas, MX (CH). While this variation in T_{max} explained only a small fraction of the variation in Bioclim variables among sites (0.0016% of the variation in PC1 and 0.00015% of the variation in PC2), previous studies have shown T_{max} to be correlated with adult heat tolerance (CT_{max}) among many species of *Drosophila* (Kellermann et al., 2012). In contrast to this previous work, our populations of *D. melanogaster* showed no significant relationship between adult heat tolerance and T_{max} (Fig. S4). However, the embryonic life stage exhibited a different pattern than the adults, and the relationship between embryonic heat tolerance and T_{max} was distinct between temperate and tropical regions (Fig. 3; ANCOVA, $F_{1,4}$ = 10.26, P = 0.0328). Among temperate populations there was a 6°C range in T_{max}, but this produced no correlated response in the thermal tolerance of embryos (Fig. 3). But among tropical populations, the approximate 4°C range in T_{max} corresponded to a positive relationship between embryonic thermal tolerance and T_{max} (Fig. 3).

Embryonic thermal tolerance in F1 progeny from Chiapas x Vermont

Offspring from reciprocal genetic crosses between the most heat tolerant tropical genotype (CH) and the least heat tolerant temperate genotype (VT-12) had thermal tolerances that closely resembled that of the heat tolerant CH genotype, regardless of the direction of the cross (Fig. 4). Embryonic LT_{50}s of F1 progeny of both crosses (CH♀ x VT♂ = 35.83°C and VT♀ x CH♂ = 35.80°C) were statistically indistinguishable from the LT_{50} of CH (36.24°C) but significantly higher than the LT_{50} of VT-12 (34.23°C; Fig. 4).

Discussion

Despite the potential for thermal adaptation across the broad range of thermal habitats represented in this study, our data suggest that thermal selection does not act equally across life stages in *D. melanogaster*. Rather, we provide evidence of adaptive variation in upper thermal limits in the thermally sensitive and immobile embryonic life stage but not in the more thermally tolerant and mobile adult stage. This is perhaps not surprising, given that lower thermal tolerance in early embryos translates into smaller thermal safety margins. Thus, we predict that embryos encounter lethal temperatures more frequently than adults, particularly because embryos lack the ability to behaviorally avoid thermally stressful conditions, and this likely drives divergence in embryonic thermal tolerance between temperate and tropical populations.

163 Recent estimates of divergence in adult thermal tolerance among populations of *D.*
164 *melanogaster* have brought into question the degree of adaptive potential in upper thermal limits
165 in this species, as comparisons of populations across latitude have yielded mixed results
166 depending on assay methods (Sgro et al., 2010) and the laboratory in which thermal tolerance
167 was measured (Hoffmann et al., 2002; Hoffmann, 2010; Buckley and Huey, 2016). Our estimates
168 of *D. melanogaster* adult male CT_{max} are consistent with previous reports (Gilchrist et al., 1997;
169 Chown et al., 2009; Kellermann et al., 2012), and while we report novel findings on the
170 adaptation of embryonic thermal tolerance, our results are not unprecedented. Coyne et al.
171 (Coyne et al., 1983) reported a similar discrepancy in thermal adaptation between mobile and
172 immobile life stages among populations of *Drosophila pseudoobscura*—pupal thermal tolerance,
173 but not adult thermal tolerance, was higher in populations from warmer locations. Thus,
174 comparisons of adult (mobile stage) thermal tolerance among populations may not accurately
175 predict the adaptive potential of upper thermal limits. Moreover, the interplay of population
176 genetic factors in natural populations of *D. melanogaster* suggest that this species harbors a high
177 level of genetic diversity (Karasov et al., 2010) and that natural selection has led to allelic
178 divergence among populations across the genome (Fabian et al., 2012). In light of these trends in
179 population genomics, and the adaptive variation in embryonic thermal tolerance presented in this
180 study, it seems probable that there is significant natural variation of upper thermal limits in *D.*
181 *melanogaster* but that this variation may only be revealed in the embryonic and other immobile
182 life stages.
183 It is important to note that lab selection experiments in *D. melanogaster*, *Escherichia coli*,
184 and marine copepods (*Tigriopus californicus*) that imposed strong selection on thermal tolerance
185 reported significant potential for adaptation of upper thermal limits, but the response to selection
186 eventually plateaued after many generations, presumably when standing genetic diversity had
187 been exhausted (Huey et al., 1991; Gilchrist et al., 1997; Gilchrist and Huey, 1999; Rudolph et
188 al., 2010; Kelly et al., 2012; Hangartner and Hoffmann, 2015). Thus, while there may likely be
189 potential for adaptation of upper thermal limits, responses to thermal selection may not occur
190 indefinitely in the absence of new mutational variation.
191 This study characterizes thermal tolerance among populations that span a large portion of
192 the *D. melanogaster* biogeographic range in the northern hemisphere, and while there is clear
193 evidence of adaptation of embryonic thermal tolerance between temperate and tropical regions,
194 the patterns of thermal adaptation are not consistent within each region. Tropical embryos
195 sampled from locations with higher T_{max} showed higher thermal tolerances, yet temperate
196 populations did not follow this trend. Why were there no observed differences in embryonic
197 thermal tolerance among temperate populations when temperate sites spanned a broader range of
198 thermal habitats than tropical populations? It is possible that gene flow between Vermont,
199 Indiana, and North Carolina overwhelms local adaptation, but recent studies show evidence of
200 adaptive divergence among *D. melanogaster* populations in eastern North America (Fabian et al.,
201 2012; Bergland et al., 2016; Machado et al., 2016). Therefore, a more likely explanation is that
202 seasonal fluctuations in the activity of temperate populations (Cogni et al., 2014; Bergland et al.,

203 2014), may limit the frequency at which temperate embryos encounter thermal selection. In
204 addition, spatial and temporal microclimatic variability in temperate sites may provide more
205 choices for females to lay their eggs at permissive temperatures (Allemand and David, 1976;
206 Dahlgaard et al., 2001; Huey and Pascual, 2009; Dillon et al., 2009). We also note that our data
207 constitute thermal tolerances of multiple isofemale lines from each of the three temperate sites
208 but just one isofemale line from each of the five tropical sites. Thus, we have not captured the
209 full range of genetic variation at each tropical site. The fact that the tropical flies originated from
210 geographically distant locations suggests that our observed differences in embryonic thermal
211 tolerance between temperate and tropical populations represent consistent patterns of thermal
212 adaptation. However, our observation that embryonic thermal tolerance positively correlates with
213 maximum temperature at tropical sites is a result that warrants further investigation. It remains to
214 be determined whether or not this pattern will hold when a greater sample of genetic diversity is
215 surveyed at these topical locations.
216 Investigations into the genetic basis of variation in thermal tolerance in natural
217 populations have yielded somewhat conflicting results, depending on the species (Pereira et al.,
218 2014; Sato et al., 2015) and the way thermal tolerance was measured (Gilchrist and Huey, 1999;
219 Mitchell and Hoffmann, 2010; Hangartner and Hoffmann, 2015). In this study, embryonic LT_{50}
220 spanned a range of approximately 2°C among all genotypes, and crosses between genotypes at
221 the high and low ends of this range produced F1 progeny that resembled the phenotype of the
222 heat-tolerant parent. This result indicates phenotypic dominance of heat-tolerant alleles, at least
223 for Chiapas vs. Vermont-12 embryos. This is a surprising result for two reasons. First, this
224 inheritance pattern is not expected because thermal physiological traits are complex and depend
225 on multiple interacting factors, such as lipid membrane composition (Dahlhoff and Somero,
226 1993; Cooper et al., 2012; Cooper et al., 2014), protein stability (Lockwood and Somero, 2012;
227 Fields et al., 2015; Leuenberger et al., 2017), and transcriptional and translational responses
228 (Leemans et al., 2000; Lockwood et al., 2010; Tomanek and Zuzow, 2010; Chen et al., 2015;
229 Kristensen et al., 2016). Accordingly, previous work has shown that *D. melanogaster* adult and
230 larval thermal tolerance are quantitative traits influenced by at least 7 gene loci (Morgan and
231 Mackay, 2006; Sambucetti et al., 2013). Similarly in species of marine invertebrates, thermal
232 tolerance appears to have a polygenic basis, as hybrids often exhibit thermal tolerances that are
233 intermediate between the parents (Braby and Somero, 2006). But this is not the case for *D.*
234 *melanogaster* embryonic thermal tolerance as we have characterized it here. Second, because we
235 examined early *D. melanogaster* embryos at 0 – 1 h post-fertilization (hpf), and zygotic genome
236 activation has been reported to not begin until approximately 2 hpf in this species (Tadros and
237 Lipshitz, 2009; Blythe and Wieschaus, 2015), we expected thermal tolerance to be determined by
238 maternal effects. Instead, we found embryonic thermal tolerance to match that of the Chiapas
239 strain regardless of maternal genotype. This suggests that either (1) the zygotic genome is being
240 activated earlier than expected in response to heat shock (Graziosi et al., 1980), which would
241 reveal adaptive variation in zygotic gene expression, or (2) that the effect is mediated at the level
242 of the chromosomes, perhaps due to thermally-induced DNA damage (Yao and Somero, 2012)

243 that differentially affects different genotypes (Svetec et al., 2016). Either way, the unknown
244 genetic basis of embryonic thermal tolerance warrants future study.
245
246 **Materials and methods**
247 **Fly strains**
248 We obtained 19 isofemale genetic lines that were collected from temperate locations in the USA
249 as a generous gift from B.S. Cooper and K.L. Montooth: 6 lines from Raleigh, NC (NC); 5 lines
250 from Beasley Orchard, IN (IN); and 8 lines from East Calais, VT (VT). The creation of these
251 lines has been previously described (Cooper et al., 2014). We obtained 5 isofemale lines from the
252 Drosophila Species Stock Center at the University of California, San Diego that were collected
253 from tropical locations around the world: 1 line each from Accra, Ghana (GH); Mumbai, India
254 (MU); Guam, USA (GU); Chiapas, Mexico (CH); and Monkey Hill, St. Kitts (SK). Geographic
255 coordinates of collection locations and stock numbers of isofemale lines are provided in
256 Supplementary Table S1. We maintained flies under common-garden conditions on cornmeal-
257 yeast-molasses medium at 25°C on a 12:12 light cycle for at least two generations prior to
258 measuring thermal tolerance.
259
260 **Adult critical thermal maximum (CT_{max})**
261 We assayed thermal tolerance of adult male flies by measuring the temperature at which flies
262 incurred a loss of motor response along a heat ramp—i.e., the critical thermal maximum (CT_{max}).
263 3 to 5 day-old adult male flies were individually placed into glass vials with rubber stoppers and
264 submerged in a water bath that was programmed to increase 0.1°C min^{-1} upwards from 25°C. We
265 chose this rate of temperature increase based on previously published studies (Chown et al.,
266 2009; Sgro et al., 2010; Kellermann et al., 2012) and to mimic the variable thermal environments
267 that flies encounter in nature (Terblanche et al., 2011). Flies were regularly checked for
268 responsiveness along the heat ramp by gently tapping the vial, and the temperature at which a fly
269 lost the ability to move was recorded. We scored CT_{max} for each genotype via a least-squares
270 regression model of the logistic equation among 10 flies per genotype and extrapolated CT_{max}
271 from the inflection points of the logistic curves. We conducted these curve fitting analyses in
272 GraphPad Prism 7 for Mac OS X (GraphPad Software, La Jolla, CA).
273
274 **Embryonic thermal tolerance (LT_{50})**
275 We assayed embryonic thermal tolerance by measuring survival (hatching success) of early stage
276 embryos, 0 to 1 h post-fertilization, exposed to a 45-minute heat treatment across a range of
277 temperatures, from 25°C to 42°C. We chose this assay method because embryos do not possess
278 behavioral characteristics that would permit the assessment of thermal tolerance via CT_{max}, and
279 measuring hatching success allowed us to overcome this obstacle. Previous studies conducted in
280 a wide range of species have shown that temperature adaptive variation in survival following
281 heat stress follows the same trends as CT_{max} (Huey et al., 1991; Gilchrist et al., 1997; Stillman
282 and Somero, 2000; Somero, 2002; Stenseng et al., 2005; Braby and Somero, 2006; Miller and

283 Stillman, 2012; Dowd and Somero, 2013). We designed our heat treatments to mimic sudden
284 increases in temperature that frequently occur in nature where the temperature of necrotic fruit
285 can increase rapidly on hot days (Feder et al., 1997; Terblanche et al., 2011). 3 to 5 day-old adult
286 flies were allowed to mate and lay eggs on grape juice agar plates for 1 h at 25°C. Egg plates
287 were then wrapped in Parafilm, submerged in a water bath, and heat shocked for 45 minutes. The
288 rate of temperature change averaged +0.4°C min^{-1}. This rate of increase is within the range of
289 measured rates of change in nature (Feder et al., 1997). Following heat shock, 20 eggs were
290 transferred on a piece of grape juice agar to fresh food vials and placed at 25°C. Hatching
291 success was scored as the proportion of larvae that successfully hatched by 48 h. We conducted 4
292 to 6 replicate treatments at each of 9 temperatures (25°C, 28°C, 30°C, 32°C, 34°C, 36°C, 38°C,
293 40°C, and 42°C) for each genotype. We used these data to calculate the lethal temperature at
294 which 50% of the embryos failed to hatch (LT_{50}) via a least-squares regression model of the
295 logistic equation. In our logistic model, we allowed the y-intercept to vary between 0 and 1 and
296 extrapolated the LT_{50} from the inflection point of the logistic curve fit. This approach allowed us
297 to infer thermal tolerance independently from other confounding factors that may influence the
298 measurement of hatching success, such as the presence of unfertilized eggs. We conducted these
299 curve fitting analyses in GraphPad Prism 7.
300
301 **PCA of bioclimatic variables**
302 We examined the correspondence of adult and embryonic thermal tolerance to local climate
303 conditions by examining the variation among our collection sites in 19 bioclimatic (Bioclim)
304 variables. Bioclim variables represent biologically meaningful climate predictors that are derived
305 from monthly temperature and rainfall data from the years 1960 to 1990 (Busby, 1991). We
306 downloaded Bioclim variables from the WorldClim database (Hijmans et al., 2005)
307 (www.worldclim.org) that corresponded to the GPS coordinates of the collection sites of each
308 population (see Table S1). Because Bioclim variables tend to covary, we used a principal
309 components analysis (PCA) to characterize the major axes of variation in climate among our
310 sites. We then conducted a multiple least-squares regression analysis to assess the degree to
311 which variation in thermal tolerance could be explained by the primary principal components of
312 these climate data. We conducted these analyses in R version 3.3.2 (Team, 2016).
313
314 **Statistical comparisons of thermal tolerance and thermal safety margins**
315 We compared adult (CT_{max}) and embryonic (LT_{50}) thermal tolerances among temperate sites (VT,
316 IN, and NC) and all tropical sites pooled together (CH, SK, GH, MU, and GU) with ANOVA.
317 Pairwise differences were assessed with Tukey's multiple comparison post-hoc test. We report
318 our data as mean values with 95% confidence intervals. We calculated thermal safety margins as
319 the difference between thermal tolerance (adult CT_{max} or embryo LT_{50}) and maximum
320 temperature of the warmest month (T_{max}) at each site (see above for description of Bioclim data).
321 Fine-scale spatial temperature data are not available for these collection sites, but while T_{max} may
322 not perfectly match the thermal environment experienced by flies, variation in T_{max} should reflect

relative differences in the thermal environments among locations. We compared thermal safety margins across latitude, as in Kellerman et al. (Kellermann et al., 2012), between adults and embryos using non-linear least-squares regression followed by the extra sum-of-squares F-test to compare model fits. In a separate analysis, we assessed the main effects of region (temperate vs. tropical), life stage (adult vs. embryo), and their interaction on thermal safety margins via a 2-way ANOVA. Least-squares linear regression was used to assess the relationship between embryonic thermal tolerance and significant climate variables. ANCOVA was used to assess the difference in slopes of regression lines fit to data from temperate vs. tropical sites. We assessed the genetic basis of differences in embryonic thermal tolerance by conducting reciprocal crosses between the two parental strains that had the highest and lowest LT_{50}, Chiapas, MX (CH) and Vermont, USA strain #12 (VT-12), respectively, and measured thermal tolerance of F1 progeny. We used logistic models to fit the hatching success data, as described above, and compared LT_{50}s of the parental strains and their F1 progeny by an extra sum-of-squares F-test of the extrapolated LT_{50}s. We conducted these analyses in GraphPad Prism 7.

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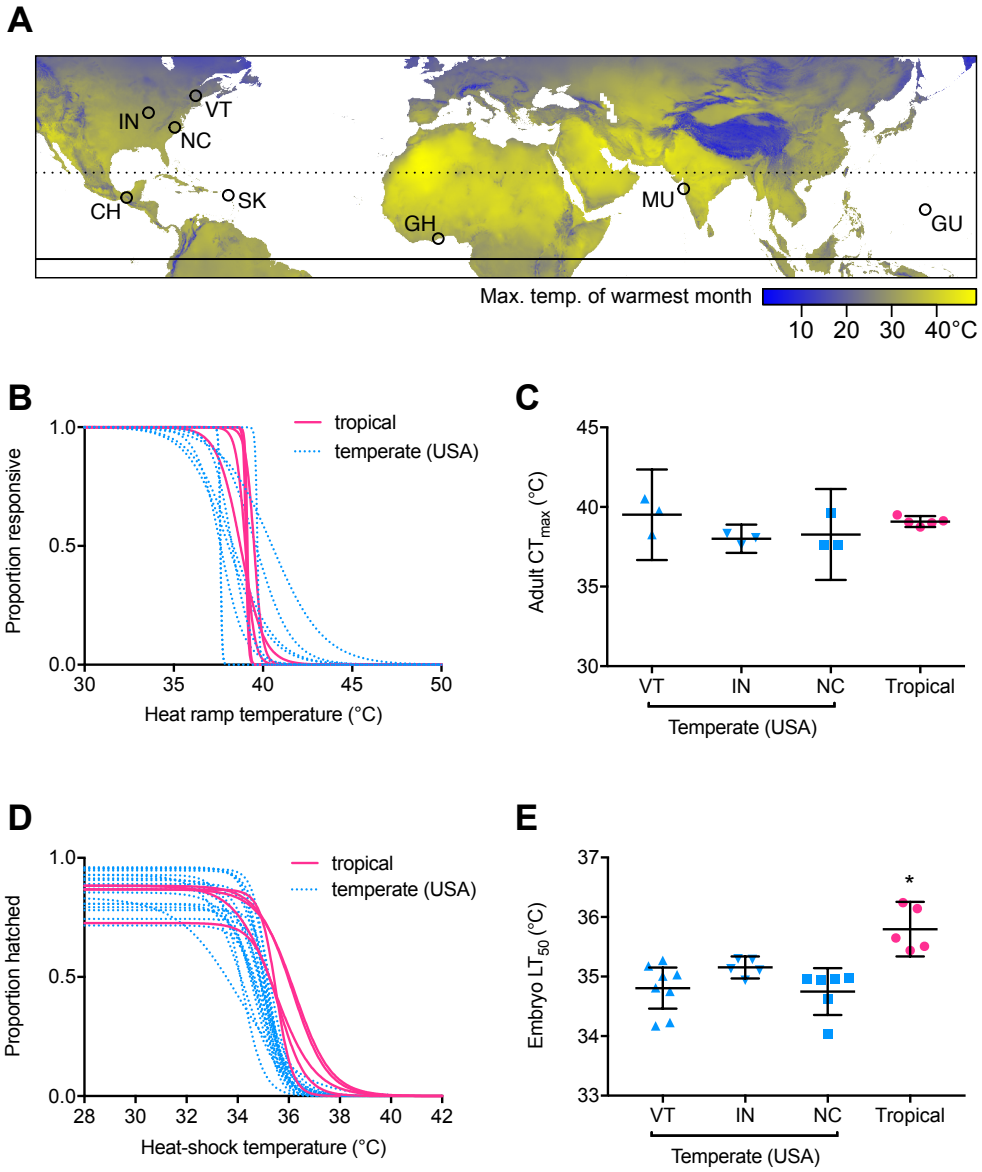
556 **Figures and Tables**



557

558 **Figure 1.**

559 **Flies from different thermal environments around the world exhibited differences in**
560 **embryonic thermal tolerance but not adult thermal tolerance.**

561 (**A**) Sampling locations of isofemale lines. Color indicates maximum temperature of warmest
562 month (T_{max}), extrapolated from the WoldClim database. Tropical populations lie between the
563 Equator (solid horizontal line) and the Tropic of Cancer (dotted horizontal line) and were
564 sampled in Chiapas, Mexico (CH), Saint Kitts (SK), Accra, Ghana (GH), Mumbai, India (MU),
565 and Guam (GU). Temperate populations lie north of the Tropic of Cancer and were sampled in
566 Vermont (VT), Indiana (IN), and North Carolina (NC). (**B**) Proportion of adult male flies
567 responsive along a heat ramp (+1°C min^{-1} from 25°C). Tropical lines are indicated in solid pink.

Temperate lines are indicated in dotted blue. (**C**) Adult male CT_{max} was consistent across all populations (ANOVA, $F_{3,9} = 2.378$, $P = 0.1375$). CT_{max} extrapolated from the survival curves in B. Solid horizontal lines show means. Error bars indicate 95% confidence intervals. (**D**) Proportion of eggs successfully hatched following heat shock (45 min at indicated temperature). Tropical lines are indicated in solid pink. Temperate lines are indicated in dotted blue. (**E**) Embryonic thermal tolerance (LT_{50}) was higher in tropical lines than temperate lines (ANOVA, $F_{3,20} = 10.16$, $P = 0.0003$; Tukey's test, VT vs. IN, $q = 2.428$, $P = 0.3416$, VT vs. NC, $q = 0.4268$, $P = 0.9902$, IN vs. NC, $q = 2.666$, $P = 0.2656$, tropical vs. VT, $q = 6.909$, $P = 0.0005$, tropical vs. IN, $q = 4.04$, $P = 0.0444$, tropical vs. NC, $q = 4.04$, $P = 0.0005$). LT_{50} extrapolated from the survival curves in D. Solid horizontal lines show means. Error bars indicate 95% confidence intervals. *$P < 0.05$.

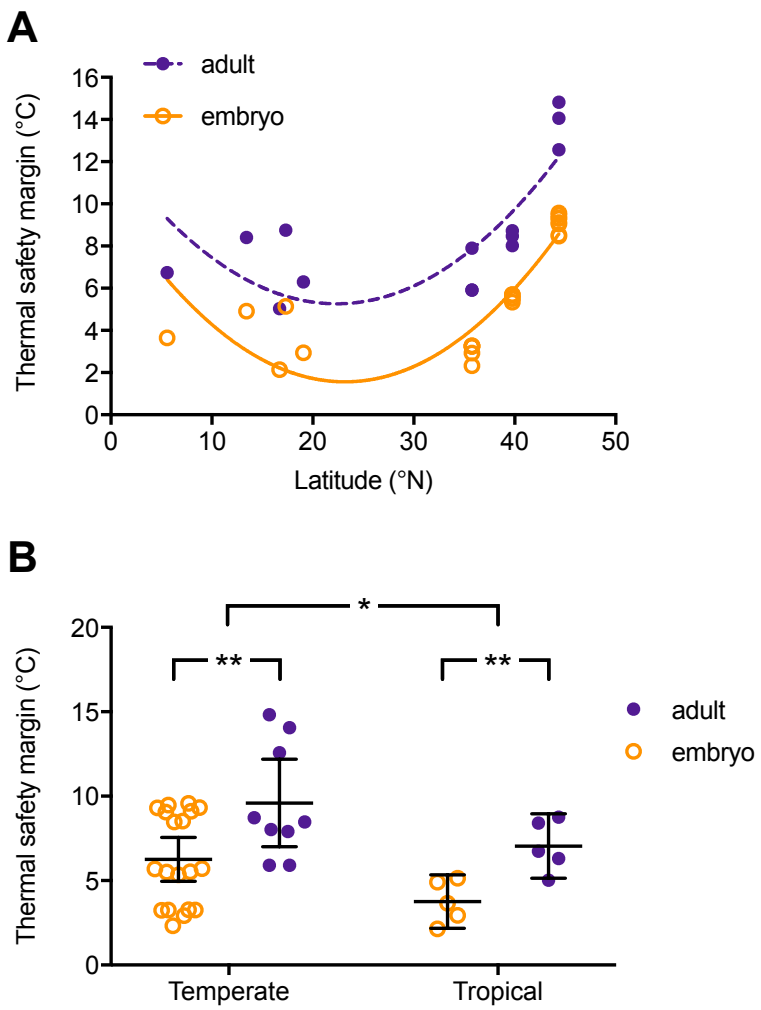
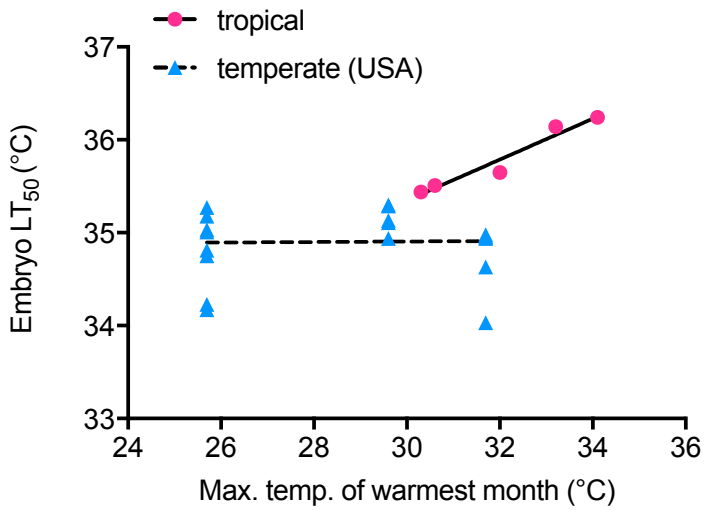


Figure 2.

Thermal safety margin differs by life stage and geographic region.

(**A**) Thermal safety margins were smaller for embryos than adults across latitude (Extra sum-of-squares F-test on fitness of two curves, $F_{3,32} = 15.95$, $P < 0.0001$; Non-linear least-squares regression, embryo, $R^2 = 0.8019$, $y = 0.01563x^2 - 0.7249x + 9.97$, adult, $R^2 = 0.6359$, $y = 0.01437x^2 - 0.6424x + 12.44$). Adults are indicated in purple filled circles with dashed purple regression line. Embryos are indicated in orange open circles with solid orange regression line. (**B**) Thermal safety margins were smaller for embryos than adults and smaller in the tropics than temperate sites (ANOVA, main effect of region, $F_{1,34} = 6.482$, $P = 0.0156$, main effect of life stage, $F_{1,34} = 11.15$, $P = 0.002$, region x life stage interaction, $F_{1,34} = 0.0006$, $P = 0.98$). Adults are indicated in purple filled circles, and embryos are indicated in orange open circles. Solid horizontal lines show means. Error bars indicate 95% confidence intervals. *$P < 0.05$, **$P < 0.01$.



596
597 **Figure 3.**
598 **Embryonic thermal tolerance and maximum habitat temperature (T_{max}) by region.**
599 Embryonic thermal tolerance was positively correlated with T_{max} among tropical populations
600 (Least-squares regression, $R^2 = 0.9478$, $P = 0.0051$, $y = 0.2199x + 28.75$) but not temperate
601 populations (Least-squares regression, $R^2 = 0.0015$, $P = 0.9751$, $y = 0.00282x + 34.82$). Tropical
602 genotypes are indicated in pink circles, with a solid black regression line fit. Temperate
603 genotypes are indicated in blue triangles, with a dashed black regression line fit.
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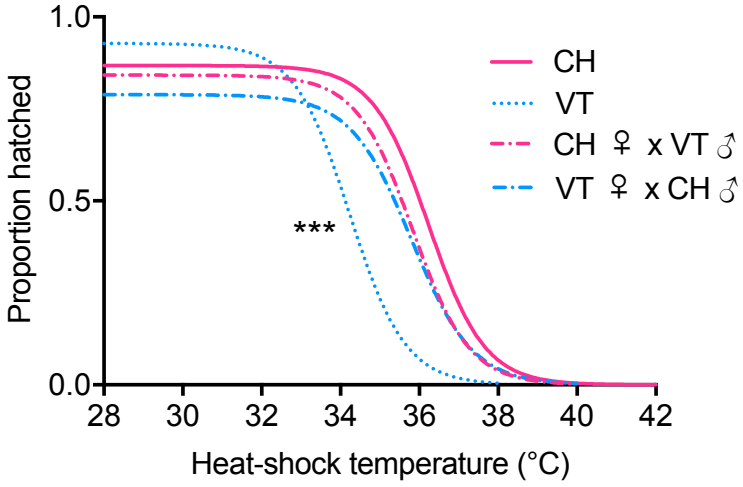

606
607 **Figure 4.**
608 **F1 progeny from tropical x temperate parents have high embryonic heat tolerance.**
609 Proportion of eggs successfully hatched following heat shock (45 min at indicated temperature)
610 among two parental genotypes that had the highest and lowest LT_{50} of all strains in this study,
611 CH (Chiapas, Mexico) and VT-12 (Vermont, USA), respectively, along with F1 progeny from
612 reciprocal crosses of these two parental lines, CH♀ x VT♂ and VT♀ x CH♂ (♀ = dam; ♂ = sire).
613 Note that VT-12 is labeled "VT" in the legend. LT_{50}: CH = 36.24°C, VT-12 = 34.23°C, CH♀ x
614 VT♂ = 35.83°C, VT♀ x CH♂ = 35.80°C (Logistic model, Extra sum-of-squares F-test on lower
615 LT_{50} of VT-12, $F_{3,166} = 6.695$, ***$P = 0.0003$).
616
617

618 **Supplemental Table S1.**
619 **Stock information, collection locales, and thermal tolerance data for isofemale lines used in**
620 **this study.**

Stock No.	Locale	State/Country	Lat. (°N)	Long. (°E)	Adult CT_{max} (°C)	Embryo LT_{50} (°C)
VTECK_2	East Calais	Vermont, USA	44.3664	-72.4300	39.69	35.18
VTECK_4	East Calais	Vermont, USA	44.3664	-72.4300	-	35.03
VTECK_5	East Calais	Vermont, USA	44.3664	-72.4300	38.27	34.75
VTECK_8	East Calais	Vermont, USA	44.3664	-72.4300	-	34.81
VTECK_9	East Calais	Vermont, USA	44.3664	-72.4300	-	35.27
VTECK_10	East Calais	Vermont, USA	44.3664	-72.4300	40.52	34.17
VTECK_12	East Calais	Vermont, USA	44.3664	-72.4300	-	34.23
VTECK_14	East Calais	Vermont, USA	44.3664	-72.4300	-	35.01
BEA_5	Beasley Orchard	Indiana, USA	39.7637	-86.4766	-	35.13
BEA_16	Beasley Orchard	Indiana, USA	39.7637	-86.4766	37.62	35.11
BEA_21	Beasley Orchard	Indiana, USA	39.7637	-86.4766	38.33	34.94
BEA_32	Beasley Orchard	Indiana, USA	39.7637	-86.4766	-	35.30
BEA_36	Beasley Orchard	Indiana, USA	39.7637	-86.4766	38.07	35.29
RFM_4	Raleigh	North Carolina, USA	35.7636	-78.6627	-	34.98
RFM_6	Raleigh	North Carolina, USA	35.7636	-78.6627	-	34.95
RFM_16	Raleigh	North Carolina, USA	35.7636	-78.6627	-	34.03
RFM_19	Raleigh	North Carolina, USA	35.7636	-78.6627	39.60	34.63
RFM_34	Raleigh	North Carolina, USA	35.7636	-78.6627	37.61	34.96
RFM_48	Raleigh	North Carolina, USA	35.7636	-78.6627	37.61	34.94
14021-0231.22	Chiapa de Corzo	Chiapas, Mexico	16.7022	-93.0081	39.13	36.24
14021-0231.34	Monkey Hill	St. Kitts	17.3240	-62.7250	39.05	35.44
14021-0231.182	Accra	Ghana	5.5557	-0.1963	38.74	35.65
14021-0231.45	Mumbai	Maharashtra, India	19.0760	72.8777	39.50	36.14
14021-0231.198	Guam	Guam, USA	13.4443	144.7937	39.01	35.51

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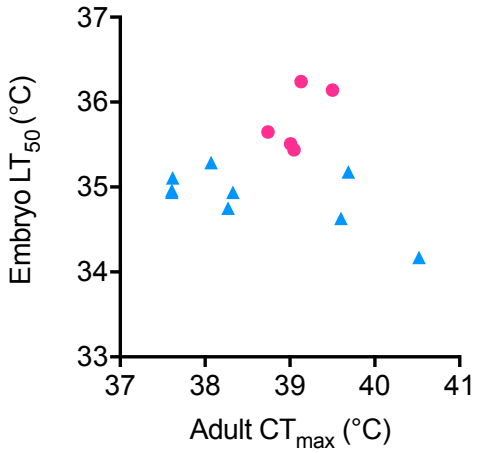
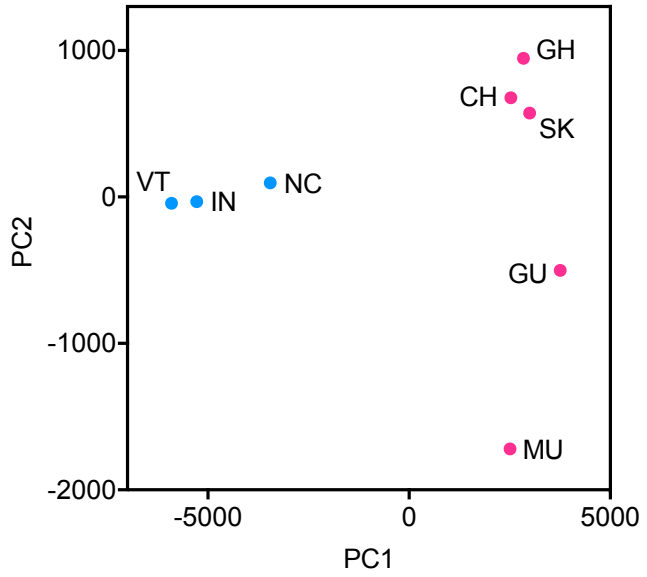
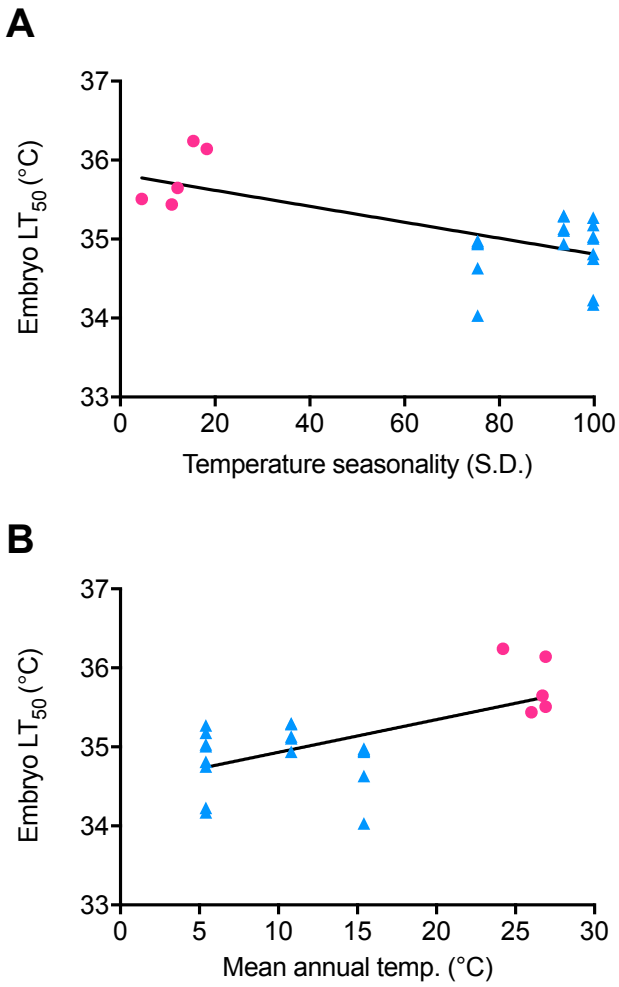


Supplemental Figure S1.
No significant relationship between adult CT_{max} and embryo LT_{50}.
Adult thermal tolerance and embryonic thermal tolerance were not correlated among all isofemale lines (Least-squares linear regression, $R^2 = 0.0005$). Tropical isofemale lines are shown in pink circles and temperate isofemale lines are shown in blue triangles.



Supplemental Figure S2.

Principal components analysis of 19 Bioclim variables at each of the 8 collection sites. Plotted are scores of the first two principle components (PC1 vs. PC2), which describe the major axes of variation in 19 bioclimatic variables among the collection sites. PC1 and PC2 explained 95.6% and 4%, respectively, of the variation in climate among sites. Temperature seasonality was the top component loading on PC1, explaining 98% of the variation in PC1, and annual precipitation was the top component loading on PC2, explaining 49% of the variation in PC2. Temperate sites are shown in blue and tropical sites in pink.



642
643 **Supplemental Figure S3.**
644 **Relationship between embryonic thermal tolerance and (A) temperature seasonality and**
645 **(B) mean annual temperature.**
646 (**A**) Temperature seasonality is the standard deviation in temperature (°C) over the course of the
647 year, averaged across the years 1960 to 1990 for each of the sites. Solid black line represents the
648 regression line fit, which had a significantly negative slope (Least-squares linear regression, R^2 =
649 0.42, P = 0.0006, y = -0.01x + 35.82). (**B**) Mean annual temperature is the average yearly
650 temperature from 1960 to 1990 for each of the sites. Solid black line represents the regression
651 line fit, which had a significantly positive slope (Least-squares linear regression, R^2 = 0.37, P =
652 0.0016, y = 0.041x + 34.52). In both A and B, tropical isofemale lines are shown in pink circles
653 and temperate isofemale lines are shown in blue triangles.
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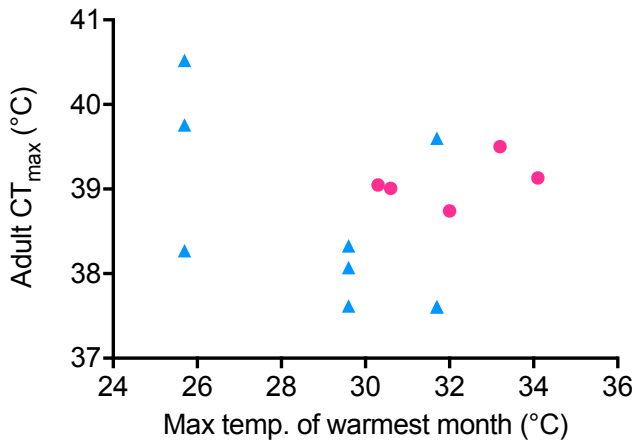

Supplemental Figure S4.
No significant relationship between adult thermal tolerance (CT_{max}) and maximum habitat temperature (T_{max}).
Variation in adult thermal tolerance showed no correspondence to variation in T_{max} among all collection sites (Least-squares linear regression, $R^2 = 0.055$). Tropical isofemale lines are shown in pink circles and temperate isofemale lines are shown in blue triangles.