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

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Summary

Many terrestrial ectothermic species exhibit limited variation in upper thermal tolerance across latitude. However, these trends may not signify limited adaptive capacity to increase thermal tolerance in the face of climate change. Instead, thermal tolerance may be similar among populations because behavioral thermoregulation by mobile organisms or life stages may buffer natural selection for thermal tolerance. We compared thermal tolerance of adults and embryos among natural populations of *Drosophila melanogaster* from a broad range of thermal habitats around the globe to assess natural variation of thermal tolerance in mobile vs. immobile life stages. We found no variation among populations in adult thermal tolerance, but embryonic thermal tolerance was higher in tropical than in temperate regions. Average maximum temperature of the warmest month of the year predicted embryonic thermal tolerance in tropical but not temperate sites. 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 thermal tolerance that matched that of tropical embryos, suggesting phenotypic dominance of heat-tolerant alleles. Together our findings demonstrate that embryonic thermal tolerance readily evolves and suggest that selection for thermal tolerance may be limited in adults. Further, our results suggest that thermal traits should be measured across life stages in order to better predict adaptive limits.

Key words: *Drosophila*, embryo, heat tolerance, phenotypic dominance, thermal adaptation, thermal safety margin

33 **Introduction**

34 Extreme temperatures, which may be encountered at the edge of a species' geographic range
35 (Hilbish *et al.* 2010) or episodically during the hottest or coldest days of the year (Hoffmann
36 2010; Kingsolver, Diamond & Buckley 2013; Dowd, King & Denny 2015; Buckley & Huey
37 2016), can cause populations to experience mortality (Helmuth *et al.* 2002; Denny, Miller &
38 Harley 2006) and ultimately lead to thermal adaptation (Lenski & Bennett 1993; Mongold,
39 Bennett & Lenski 1999; Hangartner & Hoffmann 2015). However, recent work suggests that
40 thermal adaptation of upper thermal limits might be evolutionarily constrained (Hoffmann,
41 Chown & Clusella-trullas 2013; Schou *et al.* 2014; Hangartner & Hoffmann 2015; Kristensen *et*
42 *al.* 2015; van Heerwaarden, Kellermann & Sgrò 2016), such that the evolution of increased heat
43 tolerance might be a relatively slow process that takes many millions of years to achieve
44 (Kellermann *et al.* 2012). If this is the case, global climate change, which has led to rapid
45 increases in mean temperatures and the frequency of extreme thermal events (Katz & Brown
46 1992; Meehl *et al.* 2000; Cai *et al.* 2014), may cause shifts in geographic distributions (Rank &
47 Dahlhoff 2002; Burrows *et al.* 2011; Thomas *et al.* 2012; Sunday, Bates & Dulvy 2012) as
48 populations may not be able to adapt fast enough to persist in hotter environments (Jezkova &
49 Wiens 2016).

50 But thermal adaptation depends on the strength of selection (Bennett, Lenski & Mittler
51 1992; Rudolph *et al.* 2010), and studies that focus on thermal tolerance of mobile organisms or
52 life stages may overestimate the degree to which these organisms encounter thermal selection in
53 nature. In other words, thermal safety margins—i.e., the difference between upper thermal limits
54 and maximum habitat temperature—may be larger than predicted because thermal environmental
55 heterogeneity allows mobile organisms to avoid thermal extremes via behavioral

56 thermoregulation (Dillon *et al.* 2009; Gunderson & Leal 2012; Buckley, Ehrenberger &
57 Angilletta 2015; Llewelyn *et al.* 2016; Munoz *et al.* 2016). To date, there have been relatively
58 few studies that examine thermal tolerance in immobile organisms or life stages, particularly in
59 the terrestrial realm (Angilletta *et al.* 2013; MacLean *et al.* 2016), and immobile organisms may
60 represent ideal study systems to investigate the evolutionary potential of thermal tolerance. In
61 support of this conjecture, broad scale patterns of thermal tolerance are more tightly correlated
62 with habitat temperatures in marine systems than in terrestrial systems (Sunday, Bates & Dulvy
63 2011), perhaps due to the more limited range of thermal microhabitats in the marine realm
64 (Denny *et al.* 2011) that makes behavioral thermoregulation a less effective buffering mechanism.
65 Here we sought to compare adult and embryonic heat tolerance among populations of
66 fruit flies, *Drosophila melanogaster*, from a broad range of thermal habitats across the world to
67 ascertain the degree to which thermal selection has shaped the evolution of thermal tolerance
68 across immobile vs. mobile life stages. Adult thermal tolerance has been extensively studied in
69 natural populations of *D. melanogaster* (Hoffmann, Anderson & Hallas 2002; Hoffmann &
70 Weeks 2007; Sgro *et al.* 2010; Adrion, Hahn & Cooper 2015; Buckley & Huey 2016), but to a
71 large extent the thermal physiology of the early embryonic life stage of *D. melanogaster* has not
72 been characterized in natural populations (Kristensen *et al.* 2015). Studies of lab-bred *D.*
73 *melanogaster* have shown that early embryos (0 – 2 hours post-fertilization) are more thermally
74 sensitive than later stages (Walter, Biessmann & Petersen 1990), perhaps due to the reduced
75 heat-shock response in early embryos (Graziosi *et al.* 1980; Welte *et al.* 1993). Thus, we
76 compared heat tolerance of adults and early stage embryos to determine whether or not
77 differences in thermal sensitivity, as well as mobility, lead to different patterns of thermal
78 adaptation across life stages. The thermal environment of *D. melanogaster* can change rapidly

79 (+18°C h^{-1}) and reach extreme values (> 40°C) (Feder, Blair & Figueras 1997; Terblanche *et al.*
80 2011). Therefore, we designed our thermal stress experiments to mimic sudden (acute) changes
81 in temperature that are characteristic of the variable thermal environments that flies experience in
82 nature (Terblanche *et al.* 2011). We report higher embryonic thermal tolerance in tropical
83 (hotter) vs. temperate (cooler) populations but no difference in adult thermal tolerance, and thus
84 we demonstrate that selection for thermal tolerance likely varies across life stages. Moreover, our
85 data suggest that there is significant adaptive variation for upper thermal tolerance in natural
86 populations in the earliest and most thermally sensitive life stage.
87

88 **Materials and methods**

89 **Fly strains**

90 We obtained 19 isofemale genetic lines that were collected from temperate locations in the USA
91 as a generous gift from B.S. Cooper and K.L. Montooth: 6 lines from Raleigh, NC (NC); 5 lines
92 from Beasley Orchard, IN (IN); and 8 lines from East Calais, VT (VT). The creation of these
93 lines has been previously described (Cooper, Hammad & Montooth 2014). We obtained 5
94 isofemale lines from the Drosophila Species Stock Center at the University of California, San
95 Diego that were collected from tropical locations around the world: 1 line each from Accra,
96 Ghana (GH); Mumbai, India (MU); Guam, USA (GU); Chiapas, Mexico (CH); and Monkey Hill,
97 St. Kitts (SK). Geographic coordinates of collection locations and stock numbers of isofemale
98 lines are provided in Supplementary Table S1. We maintained flies under common-garden
99 conditions on cornmeal-yeast-molasses medium at 25°C on a 12:12 light cycle for at least two
100 generations prior to measuring thermal tolerance.

101 **Adult critical thermal maximum (CT_{max})**

102 We assayed thermal tolerance of adult male flies by measuring the temperature at which flies
103 incurred a loss of motor response along a heat ramp—i.e., the critical thermal maximum (CT_{max}).
104 3 to 5 day-old adult male flies were individually placed into glass vials with rubber stoppers and
105 submerged in a water bath that was programmed to increase 0.1°C min^{-1} upwards from 25°C. We
106 chose this rate of temperature increase based on previously published studies (Chown *et al.*
107 2009; Sgro *et al.* 2010; Kellermann *et al.* 2012) and to mimic the variable thermal environments
108 that flies encounter in nature (Terblanche *et al.* 2011). Flies were regularly checked for
109 responsiveness along the heat ramp by gently tapping the vial, and the temperature at which a fly
110 lost the ability to move was recorded. We scored CT_{max} for each genotype via a least-squares
111 regression model of the logistic equation among 10 flies per genotype and extrapolated CT_{max}
112 from the inflection points of the logistic curves. We conducted these curve fitting analyses in
113 GraphPad Prism 7 for Mac OS X (GraphPad Software, La Jolla, CA).

114 **Embryonic thermal tolerance (LT_{50})**

115 We assayed embryonic thermal tolerance by measuring survival (hatching success) of early stage
116 embryos, 0 to 1 h post-fertilization, exposed to a 45-minute heat treatment across a range of
117 temperatures, from 25°C to 42°C. We chose this assay method because embryos do not possess
118 behavioral characteristics that would permit the assessment of thermal tolerance via CT_{max}, and
119 measuring hatching success allowed us to overcome this obstacle. Previous studies conducted in
120 a wide range of species have shown that temperature adaptive variation in survival following
121 heat stress follows the same trends as CT_{max} (Huey, Patridge & Fowler 1991; Gilchrist, Huey &
122 Partridge 1997; Stillman & Somero 2000; Somero 2002; Stenseng *et al.* 2005; Braby & Somero
123 2006; Miller & Stillman 2012; Dowd & Somero 2013). We designed our heat treatments to
124 mimic sudden increases in temperature that frequently occur in nature where the temperature of

125 necrotic fruit can increase rapidly on hot days (Feder *et al.* 1997; Terblanche *et al.* 2011). 3 to 5
126 day-old adult flies were allowed to mate and lay eggs on grape juice agar plates for 1 h at 25°C.
127 Egg plates were then wrapped in Parafilm, submerged in a water bath, and heat shocked for 45
128 minutes. The rate of temperature change averaged +0.4°C min^{-1}. This rate of increase is within
129 the range of measured rates of change in nature (Feder *et al.* 1997). Following heat shock, 20
130 eggs were transferred on a piece of grape juice agar to fresh food vials and placed at 25°C.
131 Hatching success was scored as the proportion of larvae that successfully hatched by 48 h. We
132 conducted 4 to 6 replicate treatments at each of 9 temperatures (25°C, 28°C, 30°C, 32°C, 34°C,
133 36°C, 38°C, 40°C, and 42°C) for each genotype. We used these data to calculate the lethal
134 temperature at which 50% of the embryos failed to hatch (LT_{50}) via a least-squares regression
135 model of the logistic equation. In our logistic model, we allowed the y-intercept to vary between
136 0 and 1 and extrapolated the LT_{50} from the inflection point of the logistic curve fit. This
137 approach allowed us to infer thermal tolerance independently from other confounding factors
138 that may influence the measurement of hatching success, such as the presence of unfertilized
139 eggs. We conducted these curve fitting analyses in GraphPad Prism 7.

140 **PCA of bioclimatic variables**

141 We examined the correspondence of adult and embryonic thermal tolerance to local climate
142 conditions by examining the variation among our collection sites in 19 bioclimatic (Bioclim)
143 variables. Bioclim variables represent biologically meaningful climate predictors that are derived
144 from monthly temperature and rainfall data from the years 1960 to 1990 (Busby 1991). We
145 downloaded Bioclim variables from the WorldClim database (Hijmans *et al.* 2005)
146 (www.worldclim.org) that corresponded to the GPS coordinates of the collection sites of each
147 population (see Table S1). Because Bioclim variables tend to covary, we used a principal

148 components analysis (PCA) to characterize the major axes of variation in climate among our
149 sites. We then conducted a multiple least-squares regression analysis to assess the degree to
150 which variation in thermal tolerance could be explained by the primary principal components of
151 these climate data. We conducted these analyses in R version 3.3.2 (Team 2016).

152 **Statistical comparisons of thermal tolerance and thermal safety margins**

153 We compared adult (CT_{max}) and embryonic (LT_{50}) thermal tolerances among temperate sites (VT,
154 IN, and NC) and all tropical sites pooled together (CH, SK, GH, MU, and GU) with ANOVA.
155 Pairwise differences were assessed with Tukey's multiple comparison post-hoc test. We report
156 our data as mean values with 95% confidence intervals. We calculated thermal safety margins as
157 the difference between thermal tolerance (adult CT_{max} or embryo LT_{50}) and maximum
158 temperature of the warmest month (T_{max}) at each site (see above for description of Bioclim data).
159 Fine-scale spatial temperature data are not available for these collection sites, but while T_{max} may
160 not perfectly match the thermal environment experienced by flies, variation in T_{max} should reflect
161 relative differences in the thermal environments among locations. We compared thermal safety
162 margins across latitude, as in Kellerman et al. (Kellermann *et al.* 2012), between adults and
163 embryos using non-linear least-squares regression followed by the extra sum-of-squares F-test to
164 compare model fits. In a separate analysis, we assessed the main effects of region (temperate vs.
165 tropical), life stage (adult vs. embryo), and their interaction on thermal safety margins via a 2-
166 way ANOVA. Least-squares linear regression was used to assess the relationship between
167 embryonic thermal tolerance and significant climate variables. ANCOVA was used to assess the
168 difference in slopes of regression lines fit to data from temperate vs. tropical sites. We assessed
169 the genetic basis of differences in embryonic thermal tolerance by conducting reciprocal crosses
170 between the two parental strains that had the highest and lowest LT_{50}, Chiapas, MX (CH) and

171 Vermont, USA strain #12 (VT-12), respectively, and measured thermal tolerance of F1 progeny.
172 We used logistic models to fit the hatching success data, as described above, and compared
173 LT_{50}s of the parental strains and their F1 progeny by an extra sum-of-squares F-test of the
174 extrapolated LT_{50}s. We conducted these analyses in GraphPad Prism 7.
175

176 **Results**

177 **Thermal tolerance and thermal safety margins across life stages**

178 We found no difference in adult thermal tolerance among all sites (Figs. 1B and 1C; ANOVA,
179 $F_{3,9} = 2.378$, $P = 0.1375$), with an overall mean CT_{max} (± 95% C.I.) of 38.8 ± 0.45°C. Embryonic
180 thermal tolerance (LT_{50}) did not differ among the three temperate sites but was significantly
181 higher in tropical vs. temperate embryos (Figs. 1D and 1E; ANOVA, $F_{3,20} = 10.16$, $P = 0.0003$;
182 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
183 $= 2.666$, $P = 0.2656$, tropical vs. VT, $q = 6.909$, $P = 0.0005$, tropical vs. IN, $q = 4.04$, $P = 0.0444$,
184 tropical vs. NC, $q = 4.04$, $P = 0.0005$). Overall, tropical embryos were more heat tolerant; the
185 average LT_{50} was approximately 1°C higher in tropical embryos (35.8 ± 0.45°C) than in
186 temperate embryos (34.88 ± 0.18°C). There was no significant relationship between adult CT_{max}
187 and embryo LT_{50} (Fig. S1; Least-squares linear regression, $R^2 = 0.0005$).

188 Thermal safety margins—i.e., the difference between thermal tolerance (CT_{max} or LT_{50})
189 and maximum habitat temperature (T_{max})—were consistently smaller for embryos than adults and
190 smallest in the tropics. This pattern was consistent regardless of whether thermal safety margins
191 were compared across latitude (Fig. 2A; Extra sum-of-squares F-test on fitness of two curves,
192 $F_{3,32} = 15.95$, $P < 0.0001$; Non-linear least-squares regression, embryo, $R^2 = 0.8019$, $y =$
193 $0.01563x^2 - 0.7249x + 9.97$, adult, $R^2 = 0.6359$, $y = 0.01437x^2 - 0.6424x + 12.44$) or by region

194 (temperate vs. tropical) (Fig. 2B; ANOVA, main effect of region, $F_{1,34} = 6.482$, $P = 0.0156$, main
195 effect of life stage, $F_{1,34} = 11.15$, $P = 0.002$, region x life stage interaction, $F_{1,34} = 0.0006$, $P =$
196 0.98).

197 **Climate variability and thermal tolerance**

198 While there is a consistent difference between our temperate and tropical sites in mean annual
199 temperature, VT = 5.4°C, IN = 10.8°C, NC = 15.4°C, and all tropical = 26.1 ± 1.4°C (mean ±
200 95% C.I.), a principal components analysis of 19 Bioclim variables indicated that the greatest
201 difference in climate among the collection locations was in temperature seasonality—defined as
202 the yearly standard deviation in temperature. PC1 explained 95.6% of the variation in climate
203 data among the collection sites, and this pattern largely characterized the differences in climate
204 between temperate vs. tropical sites (Fig. S2). Temperature seasonality was the variable with the
205 top component loading of PC1, explaining 98% of the variation in PC1. There was a significant
206 negative relationship between PC1 and embryonic heat tolerance (Multiple least-squares linear
207 regression with PC1 and PC2 as covariates, $F_{1,4} = 8.1474$, $P = 0.0462$), but this pattern was
208 driven by the bimodal distribution of LT_{50}s between temperate vs. tropical sites and was not
209 consistent with temperature seasonality within each geographic region (Fig. S3). PC2 explained
210 4% of the variation in climate data among collection sites, mostly characterizing the differences
211 in climate among tropical sites (Fig. S2). Annual precipitation, precipitation of the wettest
212 quarter, and precipitation of the wettest month were the top three component loadings of PC2,
213 explaining 49%, 40%, and 8% of variation in PC2, respectively. But there was no significant
214 relationship between PC2 and embryonic heat tolerance (Least-squares linear regression, $F_{1,4} =$
215 0.367, $P = 0.577$).

216 Maximum temperature of the warmest month (i.e., maximum habitat temperature or T_{max})
217 spanned a range of 8.4°C among all sites, from 25.7°C in Vermont, USA (VT) to 34.1°C in
218 Chiapas, MX (CH). While this variation in T_{max} explained only a small fraction of the variation
219 in Bioclim variables among sites (0.0016% of the variation in PC1 and 0.00015% of the
220 variation in PC2), previous studies have shown T_{max} to be correlated with adult heat tolerance
221 (CT_{max}) among many species of *Drosophila* (Kellermann *et al.* 2012). In contrast to this previous
222 work, our populations of *D. melanogaster* showed no significant relationship between adult heat
223 tolerance and T_{max} (Fig. S4; Least-squares linear regression, $R^2 = 0.055$). However, the
224 embryonic life stage exhibited a different pattern than the adults, and the relationship between
225 embryonic heat tolerance and T_{max} was distinct between temperate and tropical regions (Fig. 3;
226 ANCOVA, $F_{1,4} = 10.26$, $P = 0.0328$). Among temperate populations there was a 6°C range in
227 T_{max}, but this produced no correlated response in the thermal tolerance of embryos (Fig. 3; Least-
228 squares regression, $R^2 = 0.0015$, $P = 0.9751$, $y = 0.00282x + 34.82$). But among tropical
229 populations, the approximate 4°C range in T_{max} corresponded to a positive relationship between
230 embryonic thermal tolerance and T_{max} (Fig. 3; Least-squares regression, $R^2 = 0.9478$, $P = 0.0051$,
231 $y = 0.2199x + 28.75$).

232 **Embryonic thermal tolerance in F1 progeny from Chiapas x Vermont**

233 Offspring from reciprocal genetic crosses between the most heat tolerant tropical genotype (CH)
234 and the least heat tolerant temperate genotype (VT-12) had thermal tolerances that closely
235 resembled that of the heat tolerant CH genotype, regardless of the direction of the cross (Fig. 4).
236 Embryonic LT_{50}s of F1 progeny of both crosses (CH♀ x VT♂ = 35.83°C and VT♀ x CH♂ =
237 35.80°C) were statistically indistinguishable from the LT_{50} of CH (36.24°C) but significantly

238 higher than the LT_{50} of VT-12 (34.23°C; Fig. 4; Logistic model, Extra sum-of-squares F-test on
239 lower LT_{50} of VT-12, $F_{3,166} = 6.695$).
240
241 **Discussion**
242 Despite the potential for thermal adaptation across the broad range of thermal habitats
243 represented in this study, our data suggest that natural selection on thermal tolerance does not act
244 equally across life stages in *D. melanogaster*. Rather, we provide evidence of adaptive variation
245 in upper thermal limits in the thermally sensitive and immobile embryonic life stage but not in
246 the more thermally tolerant and mobile adult stage. This is perhaps not surprising, given that
247 lower thermal tolerance in early embryos translates into smaller thermal safety margins. Thus,
248 we predict that embryos encounter lethal temperatures more frequently than adults, particularly
249 because embryos lack the ability to behaviorally avoid thermally stressful conditions, and this
250 likely drives divergence in embryonic thermal tolerance between temperate and tropical
251 populations.
252 Recent estimates of divergence in adult thermal tolerance among populations of *D.*
253 *melanogaster* have brought into question the degree of adaptive potential in upper thermal limits
254 in this species, as comparisons of populations across latitude have yielded mixed results
255 depending on assay methods (Sgro *et al.* 2010) and the laboratory in which thermal tolerance
256 was measured (Hoffmann *et al.* 2002; Hoffmann 2010; Buckley & Huey 2016). Our estimates of
257 *D. melanogaster* adult male CT_{max} are consistent with previous reports (Gilchrist *et al.* 1997;
258 Chown *et al.* 2009; Kellermann *et al.* 2012), and while we report novel findings on the
259 adaptation of embryonic thermal tolerance, our results are not unprecedented. Coyne et al.
260 (Coyne, Bundgaard & Prout 1983) reported a similar discrepancy in thermal adaptation between

261 mobile and immobile life stages among populations of *Drosophila pseudoobscura*—pupal
262 thermal tolerance, but not adult thermal tolerance, was higher in populations from warmer
263 locations. The interplay of population genetic factors in natural populations of *D. melanogaster*
264 suggest that this species harbors a high level of genetic diversity (Karasov, Messer & Petrov
265 2010) and that natural selection has led to allelic divergence among populations across the
266 genome (Hoffmann & Weeks 2007; Fabian *et al.* 2012; Adrion *et al.* 2015). In light of these
267 trends in population genomics, and the adaptive variation in embryonic thermal tolerance
268 presented in this study, it seems probable that there is significant natural variation of upper
269 thermal limits in *D. melanogaster* but that this variation may only be revealed in the embryonic
270 and other immobile life stages.

271 It is important to note that lab selection experiments in *D. melanogaster*, *Escherichia coli*,
272 and marine copepods (*Tigriopus californicus*) that imposed strong selection on thermal tolerance
273 reported significant potential for adaptation of upper thermal limits, but the response to selection
274 eventually plateaued after many generations, presumably when standing genetic diversity had
275 been exhausted (Huey *et al.* 1991; Gilchrist *et al.* 1997; Gilchrist & Huey 1999; Rudolph *et al.*
276 2010; Kelly, Sanford & Grosberg 2012; Hangartner & Hoffmann 2015). Thus, there may likely
277 be potential for adaptation of upper thermal limits, and in natural populations greater levels of
278 standing genetic variation may be able to sustain adaptive responses to thermal selection.

279 This study characterizes thermal tolerance among populations that span a large portion of
280 the *D. melanogaster* biogeographic range in the northern hemisphere, and while there is clear
281 evidence of adaptation of embryonic thermal tolerance between temperate and tropical regions,
282 the patterns of thermal adaptation are not consistent within each region. Tropical embryos
283 sampled from locations with higher T_{max} showed higher thermal tolerances, yet temperate

284 populations did not follow this trend. Why were there no observed differences in embryonic
285 thermal tolerance among temperate populations when temperate sites spanned a broader range of
286 thermal habitats than tropical populations? It is possible that gene flow between Vermont,
287 Indiana, and North Carolina overwhelms local adaptation, but recent studies show evidence of
288 adaptive divergence among *D. melanogaster* populations in eastern North America (Fabian *et al.*
289 2012; Bergland *et al.* 2016; Machado *et al.* 2016). Therefore, a more likely explanation is that
290 seasonal fluctuations in the activity of temperate populations (Cogni *et al.* 2014), may limit the
291 frequency at which temperate embryos encounter thermal selection. In addition, spatial and
292 temporal microclimatic variability in temperate sites may provide more choices for females to
293 lay their eggs at permissive temperatures (Allemand & David 1976; Dahlgaard, Hasson &
294 Loeschcke 2001; Huey & Pascual 2009; Dillon *et al.* 2009). We also note that our data constitute
295 thermal tolerances of multiple isofemale lines from each of the three temperate sites but just one
296 isofemale line from each of the five tropical sites. Thus, we have not captured the full range of
297 genetic variation at each tropical site. The fact that the tropical flies originated from
298 geographically distant locations suggests that our observed differences in embryonic thermal
299 tolerance between temperate and tropical populations represent consistent patterns of thermal
300 adaptation. However, our observation that embryonic thermal tolerance positively correlates with
301 maximum temperature at tropical sites is a result that warrants further investigation. It remains to
302 be determined whether or not this pattern will hold when a greater sample of genetic diversity is
303 surveyed at these topical locations.

304 Investigations into the genetic basis of variation in thermal tolerance in natural
305 populations have yielded somewhat conflicting results, depending on the species (Pereira,
306 Barreto & Burton 2014; Sato *et al.* 2015) and the way thermal tolerance was measured (Gilchrist

307 & Huey 1999; Mitchell & Hoffmann 2010; Hangartner & Hoffmann 2015). In this study,
308 embryonic LT_{50} spanned a range of approximately 2°C among all genotypes, and crosses
309 between genotypes at the high and low ends of this range produced F1 progeny that resembled
310 the phenotype of the heat-tolerant parent. This result indicates phenotypic dominance of heat-
311 tolerant alleles, at least for Chiapas vs. Vermont-12 embryos. This is a surprising result for two
312 reasons. First, this inheritance pattern is not expected because thermal physiological traits are
313 complex and depend on multiple interacting factors, such as lipid membrane composition
314 (Dahlhoff & Somero 1993; Cooper *et al.* 2012, 2014), protein stability (Lockwood & Somero
315 2012; Fields *et al.* 2015; Leuenberger *et al.* 2017), and transcriptional and translational responses
316 (Leemans *et al.* 2000; Lockwood, Sanders & Somero 2010; Tomanek & Zuzow 2010; Chen,
317 Nolte & Schlotterer 2015; Kristensen *et al.* 2016). Accordingly, previous work has shown that *D.*
318 *melanogaster* adult and larval thermal tolerance are quantitative traits influenced by at least 7
319 loci (Morgan & Mackay 2006; Sambucetti *et al.* 2013). Second, because we examined early *D.*
320 *melanogaster* embryos at 0 – 1 h post-fertilization (hpf), and zygotic genome activation has been
321 reported to not begin until approximately 2 hpf in this species (Tadros & Lipshitz 2009; Blythe
322 & Wieschaus 2015), we expected thermal tolerance to be determined by maternal effects. Instead,
323 we found embryonic thermal tolerance to match that of the Chiapas strain regardless of maternal
324 genotype. This suggests that either (1) the zygotic genome is being activated earlier than
325 expected in response to heat shock (Graziosi *et al.* 1980), which would reveal adaptive variation
326 in zygotic gene expression, or (2) that the effect is mediated at the level of the chromosomes,
327 perhaps due to thermally-induced DNA damage (Yao & Somero 2012) that differentially affects
328 different genotypes (Svetec *et al.* 2016). Either way, the unknown genetic basis of embryonic
329 thermal tolerance warrants future study.

330

331 **Author's Contributions**

332 BL conceived the ideas and designed the methodology; BL, TG, and RS collected the data; BL
333 analyzed the data; BL wrote the manuscript. All authors gave final approval for publication.

334

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340

341 **Data Accessibility**

342 Fly stock information is included in Table S1, including geographical coordinates of sampling
343 locations, stock numbers, and thermal tolerance data.

344

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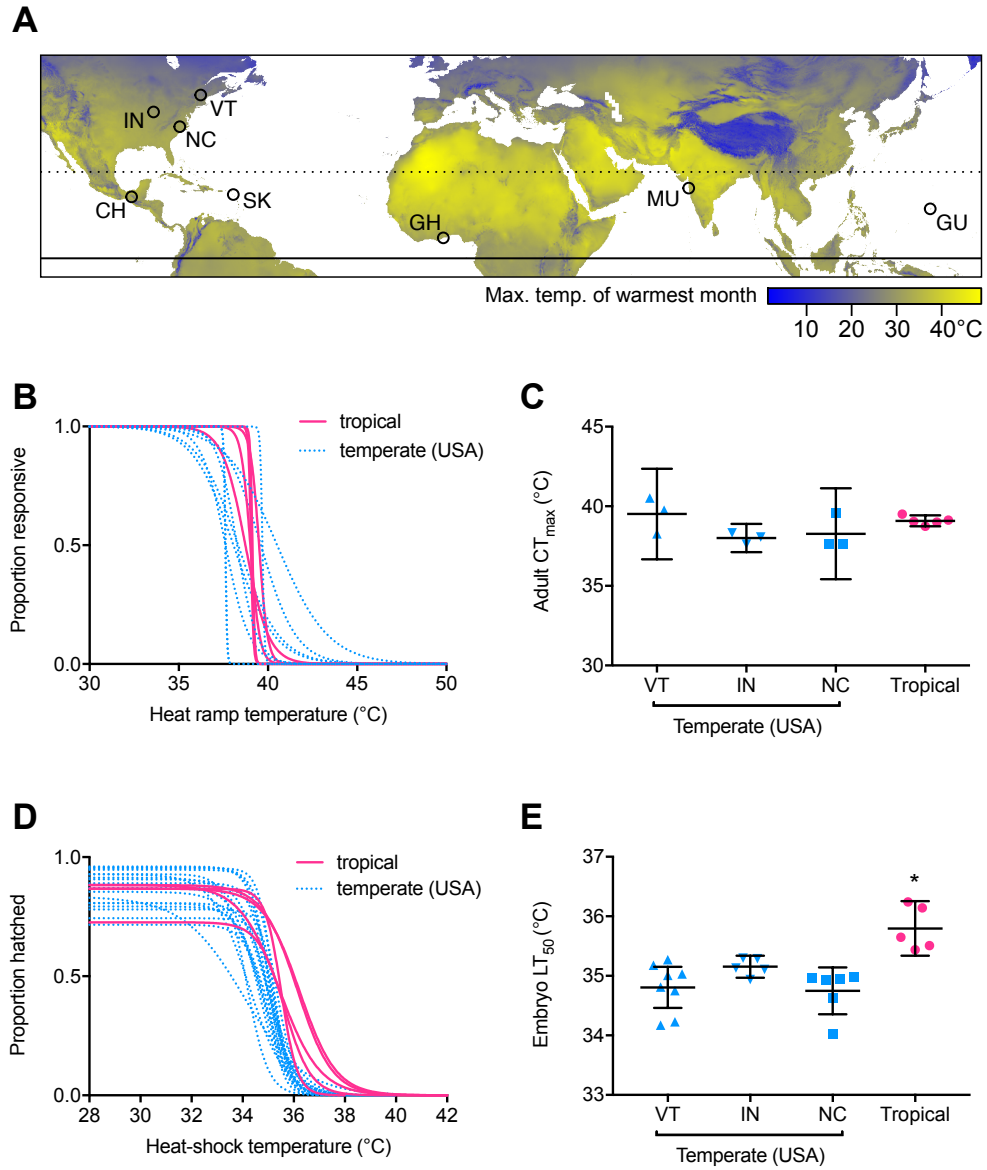
585
586

587 **Figures**



588

589 **Figure 1.**

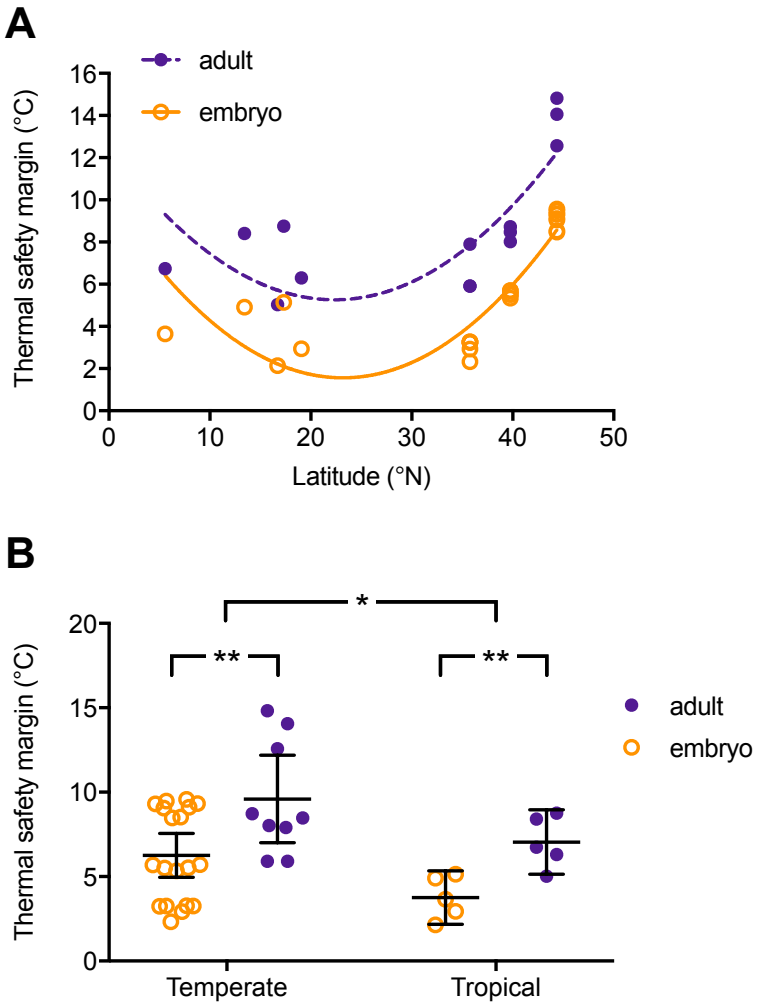
590 **Flies from different populations around the world exhibited differences in embryonic**

591 **thermal tolerance but not adult thermal tolerance.**

592 (**A**) Sampling locations of isofemale lines. Color indicates maximum temperature of warmest

593 month (T_{max}), extrapolated from the WorldClim database. Tropical populations lie between the

594 Equator (solid horizontal line) and the Tropic of Cancer (dotted horizontal line) and were
595 sampled in Chiapas, Mexico (CH), Saint Kitts (SK), Accra, Ghana (GH), Mumbai, India (MU),
596 and Guam (GU). Temperate populations lie north of the Tropic of Cancer and were sampled in
597 Vermont (VT), Indiana (IN), and North Carolina (NC). (**B**) Proportion of adult male flies
598 responsive along a heat ramp (+1°C min^{-1} from 25°C). Tropical lines are indicated in solid pink.
599 Temperate lines are indicated in dotted blue. (**C**) Adult male CT_{max} was consistent across all
600 populations (ANOVA, $F_{3,9} = 2.378$, $P = 0.1375$). CT_{max} extrapolated from the survival curves in
601 B. Solid horizontal lines show means. Error bars indicate 95% confidence intervals. (**D**)
602 Proportion of eggs successfully hatched following heat shock (45 min at indicated temperature).
603 Tropical lines are indicated in solid pink. Temperate lines are indicated in dotted blue. (**E**)
604 Embryonic thermal tolerance (LT_{50}) was higher in tropical lines than temperate lines (ANOVA,
605 $F_{3,20} = 10.16$, $P = 0.0003$; Tukey's test, VT vs. IN, $q = 2.428$, $P = 0.3416$, VT vs. NC, $q = 0.4268$,
606 $P = 0.9902$, IN vs. NC, $q = 2.666$, $P = 0.2656$, tropical vs. VT, $q = 6.909$, $P = 0.0005$, tropical vs.
607 IN, $q = 4.04$, $P = 0.0444$, tropical vs. NC, $q = 4.04$, $P = 0.0005$). LT_{50} extrapolated from the
608 survival curves in D. Solid horizontal lines show means. Error bars indicate 95% confidence
609 intervals. *$P < 0.05$.



610

611 **Figure 2.**

612 **Thermal safety margin differs by life stage and geographic region.**

613 (**A**) Thermal safety margins were smaller for embryos than adults across latitude (Extra sum-of-

614 squares F-test on fitness of two curves, $F_{3,32} = 15.95$, $P < 0.0001$; Non-linear least-squares

615 regression, embryo, $R^2 = 0.8019$, $y = 0.01563x^2 - 0.7249x + 9.97$, adult, $R^2 = 0.6359$, $y =$

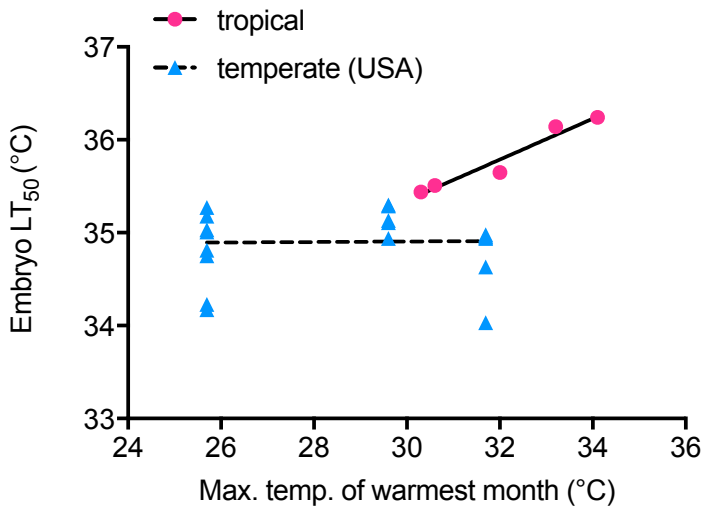
616 $0.01437x^2 - 0.6424x + 12.44$). Adults are indicated in purple filled circles with dashed purple

617 regression line. Embryos are indicated in orange open circles with solid orange regression line.

618 (**B**) Thermal safety margins were smaller for embryos than adults and smaller in the tropics than

619 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. *$P < 0.05$, **$P < 0.01$.



624

625 **Figure 3.**

626 **Embryonic thermal tolerance and maximum habitat temperature (T_{max}) by region.**

627 Embryonic thermal tolerance was positively correlated with T_{max} among tropical populations

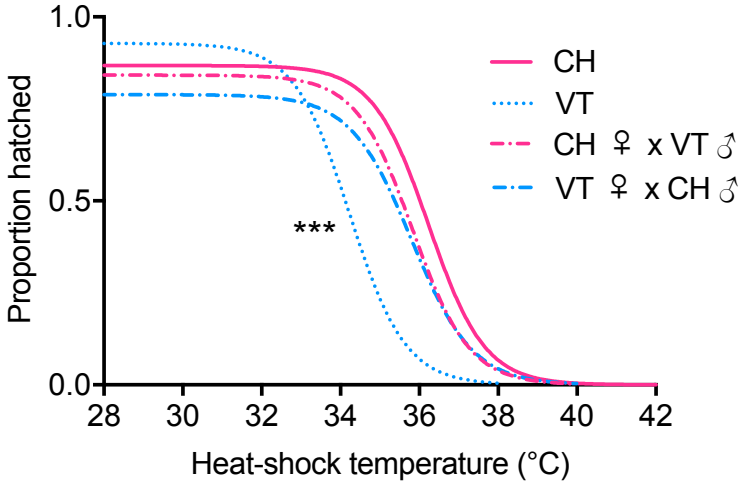
628 (Least-squares regression, $R^2 = 0.9478$, $P = 0.0051$, $y = 0.2199x + 28.75$) but not temperate

629 populations (Least-squares regression, $R^2 = 0.0015$, $P = 0.9751$, $y = 0.00282x + 34.82$). Tropical

630 genotypes are indicated in pink circles, with a solid black regression line fit. Temperate

631 genotypes are indicated in blue triangles, with a dashed black regression line fit.

632



633

634 **Figure 4.**

635 **F1 progeny from tropical x temperate parents have high embryonic heat tolerance.**

636 Proportion of eggs successfully hatched following heat shock (45 min at indicated temperature)

637 among two parental genotypes that had the highest and lowest LT_{50} of all strains in this study,

638 CH (Chiapas, Mexico) and VT-12 (Vermont, USA), respectively, along with F1 progeny from

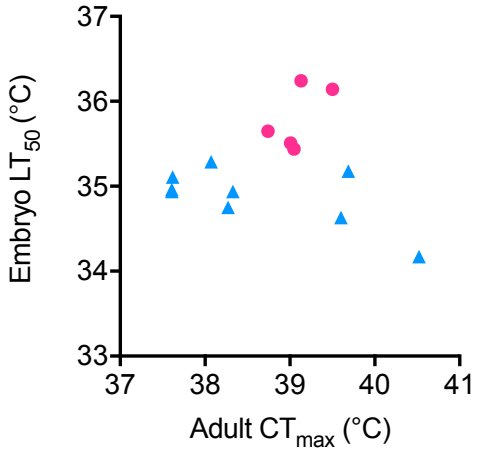
639 reciprocal crosses of these two parental lines, CH♀ x VT♂ and VT♀ x CH♂ (♀ = dam; ♂ = sire).

640 Note that VT-12 is labeled "VT" in the legend. LT_{50}: CH = 36.24°C, VT-12 = 34.23°C, CH♀ x

641 VT♂ = 35.83°C, VT♀ x CH♂ = 35.80°C (Logistic model, Extra sum-of-squares F-test on lower

642 LT_{50} of VT-12, $F_{3,166} = 6.695$, ***$P = 0.0003$).

643



644

645 **Supplemental Figure S1.**

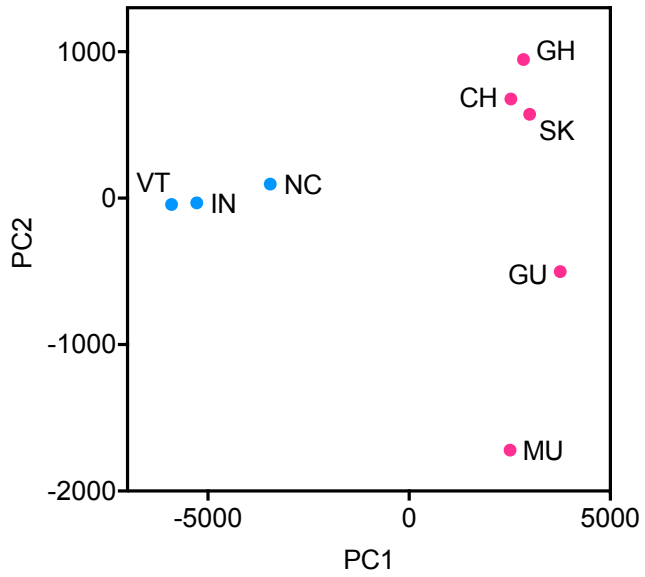
646 **No significant relationship between adult CT_{max} and embryo LT_{50}.**

647 Adult thermal tolerance and embryonic thermal tolerance were not correlated among all

648 isofemale lines (Least-squares linear regression, $R^2 = 0.0005$). Tropical isofemale lines are

649 shown in pink circles and temperate isofemale lines are shown in blue triangles.

650



651

652 **Supplemental Figure S2.**

653 **Principal components analysis of 19 Bioclim variables at each of the 8 collection sites.**

654 Plotted are scores of the first two principle components (PC1 vs. PC2), which describe the major

655 axes of variation in 19 bioclimatic variables among the collection sites. PC1 and PC2 explained

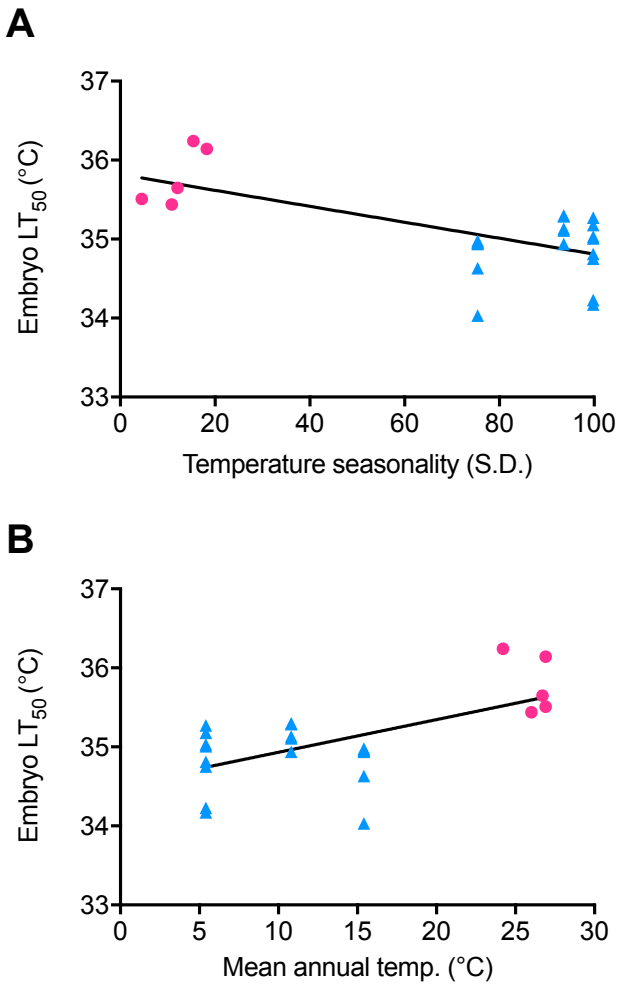
656 95.6% and 4%, respectively, of the variation in climate among sites. Temperature seasonality

657 was the top component loading on PC1, explaining 98% of the variation in PC1, and annual

658 precipitation was the top component loading on PC2, explaining 49% of the variation in PC2.

659 Temperate sites are shown in blue and tropical sites in pink.

660

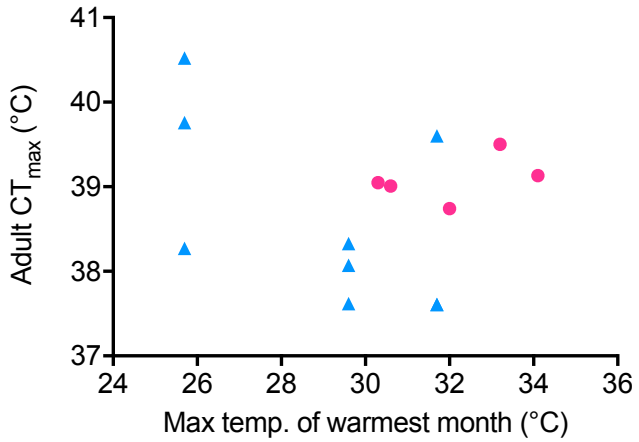


Supplemental Figure S3.

Relationship between embryonic thermal tolerance and (A) temperature seasonality and (B) mean annual temperature.

(**A**) Temperature seasonality is the standard deviation in temperature (°C) over the course of the year, averaged across the years 1960 to 1990 for each of the sites. Solid black line represents the regression line fit, which had a significantly negative slope (Least-squares linear regression, R^2 = 0.42, P = 0.0006, y = -0.01x + 35.82). (**B**) Mean annual temperature is the average yearly temperature from 1960 to 1990 for each of the sites. Solid black line represents the regression

line fit, which had a significantly positive slope (Least-squares linear regression, $R^2 = 0.37$, $P = 0.0016$, $y = 0.041x + 34.52$). In both A and B, tropical isofemale lines are shown in pink circles and temperate isofemale lines are shown in blue triangles.



674

675 **Supplemental Figure S4.**

676 **No significant relationship between adult thermal tolerance (CT_{max}) and maximum habitat**

677 **temperature (T_{max}).**

678 Variation in adult thermal tolerance showed no correspondence to variation in T_{max} among all

679 collection sites (Least-squares linear regression, $R^2 = 0.055$). Tropical isofemale lines are shown

680 in pink circles and temperate isofemale lines are shown in blue triangles.

681

682 **Tables**

683 **Supplemental Table S1.**

684 Stock information, collection locales, and thermal tolerance data for isofemale lines used in this

685 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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