Title: Different factors shape pigmentation and other physiological trait variation within and between species in the *Drosophila americana* group.

Running title: Pigmentation variation in the *D. americana* group

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Abstract

Disentangling the factors shaping adaptive trait variation is an important but challenging task. Natural pigmentation variation in *Drosophila* is one instance where studies support multiple—sometimes contradictory—proposed mechanisms for this variation, based on conflicting patterns of association with other traits and/or environmental factors. Here we systematically examined associations between pigmentation variation, a suite of environmental variables, and two other physiological traits (desiccation and UV resistance), to critically evaluate several of these proposed mechanisms. Using 3 populations from each of 3 species—*D. americana americana, D. a. texana* and *D. novamexicana*—we detected significant population- and species-level variation for all three traits and environmental preferences. Further, we found that patterns of pigmentation variation differed between evolutionary scales: within species, darker pigmentation was associated with hotter, drier, more irradiated environments, but an opposing association (i.e., lighter species in these environments) was evident between species. Weak (desiccation resistance) or no (UV resistance) associations with other trait variation suggests little evidence that pleiotropy with these traits directly shapes pigmentation variation. By simultaneously assessing multiple trait-trait and trait-environment relationships, our approach indicates pigmentation variation is likely shaped by alternative factors at different evolutionary scales, supporting different selective hypotheses at each of these scales.

Introduction

Differentiating the historical, genetic, and selective factors that cause some traits to vary locally according to environment, while other traits become fixed between lineages, is of critical importance to our understanding of how adaptations arise, spread, and persist. One hallmark of local adaptation is a consistent association between trait variation and one or more aspects of the natural environment. Classically, these associations have been assessed via the study of clines; by definition, clines exhibit spatial variation, and geographic space is frequently environmentally heterogeneous, so traits exhibiting functionally-relevant clinal variation are clear candidates for targets of local selection. Latitudinal or altitudinal clines have received particular attention in numerous systems, including *Drosophila*, *Arabidopsis thaliana*, and humans, where analyses indicate strong trait-environment associations for several physiological and other variants (Adrian et al. 2015, Fournier-Level et al. 2011, Hancock et al. 2011). Nonetheless, even among these well-characterized examples, the underlying cause of clinal variation is still not always clear, particularly when trait variation is surveyed across generalized geographic space as opposed to a specific environmental gradient. In addition, clinal analyses are often designed to identify traits that are subject to strong local adaptation—that is, that vary among populations within species. However, some ecologically important traits might instead show discrete differences between closely related species, especially when closely-related species are themselves spatially partitioned. Whether these species-level differences dominate patterns of adaptation can depend upon when variants arose in the history of lineage differentiation, whether they confer a net fitness benefit in spite of intraspecific environmental variation, and/or whether they are subject to constraints that prevent them from responding to local conditions. Studying traits that exhibit a range of variation, both between populations within species and between populations of closely related species, could therefore be invaluable in understanding how natural selection can differentially shape trait variation at different spatial and evolutionary scales.

69 In *Drosophila,* patterns of intraspecific clinal variation and species differences have unveiled
70 several traits as potential targets of environmentally-mediated selection, including cuticle pigmentation,
71 and desiccation and UV resistance. Nonetheless, in many cases the environmental factors responsible
72 for driving variation in these traits is equivocal. For example, multiple studies have shown an association
73 between pigmentation variation and latitude, including within *D. melanogaster* in Europe (David et al.
74 1985), Australia (Telonis-Scott et al. 2011), India (Munjal et al. 1997), and sub-saharan Africa (Pool and
75 Aquadro 2007, Bastide et al. 2014), as well as within *D. simulans* (Capy et al. 1988) and the *D. cardini*
76 group (Heed and Krishnamurthy 1959). However, the nature of this association varies between studies;
77 while most find increased melanization at higher latitudes, the opposite pattern (i.e., darker flies at
78 lower latitudes) has been observed in *D. melanogaster* from sub-saharan Africa (Pool and Aquadro 2007,
79 Bastide et al. 2014). Moreover, while a number of hypotheses have been proposed to explain latitudinal
80 clines based on direct physiological effects of pigmentation variation—including selection for increased
81 pigmentation at higher, colder latitudes in order to increase absorption of solar radiation (the Thermal
82 Melanism Hypothesis, or Bogert's Rule; Gaston et al. 2009)—direct empirical evidence in support of this
83 and similar hypotheses remains limited and inconclusive.

84 Alternative hypotheses propose that selection for pigmentation variation is driven by a
85 pleiotropic association with other physiological stress resistance responses, especially UV radiation
86 and/or desiccation resistance responses. For example, selection for increased UV resistance has been
87 proposed to drive increased (darker) pigmentation, however empirical data indicates that the
88 mechanistic relationship between these two traits is more complex. Darker pigmentation is associated
89 with increased UV exposure and altitude in sub-saharan *D. melanogaster* (Pool and Aquadro 2007,
90 Bastide et al. 2014), but the opposite pattern has been observed for *D. yakuba* and *D. santomea*, with
91 lighter flies exhibiting greater UV resistance (Matute and Harris 2013). Experimentally, both light
92 (*yellow*) and dark (*ebony*) mutants have decreased survivorship after UV exposure (relative to wild type),

93 although dark mutants retained significantly better UV resistance compared to light mutants (Wang et
94 al. 2008). Similar to UV resistance, pleiotropic associations with desiccation stress have also been
95 proposed as a causal factor shaping pigmentation variation, with studies showing that increased
96 desiccation resistance is correlated clinally with darker pigmentation in *D. polymorpha* (Brisson et al.
97 2005) and Indian *D. melanogaster* and *D. ananassae* (Parkash et al. 2008, Parkash et al. 2010), although
98 this relationship was not observed in *D. yakuba/D. santomea* (Matute and Harris 2013). Experimental
99 evidence for a role of melanization in desiccation resistance is also mixed. In *D. melanogaster, D.*
100 *simulans, D. subobscura, and D. pseudoobscura*, *yellow* (light pigment) mutants have lower survival after
101 desiccation stress (relative to wild type), while *ebony D. melanogaster* mutants survive significantly
102 longer than wildtype (Kalmus 1941). However, Wittkopp et al. (2010) showed that introgression of dark
103 alleles into a light background and vice versa produced no effect on desiccation resistance among
104 species in the *D. americana* group. It therefore remains unclear whether these two traits are directly
105 causally connected or simply covary due to their shared association with other variables that remain
106 unaccounted for.

107 Here, our goal was to simultaneously evaluate desiccation resistance, UV resistance, and
108 pigmentation, the relationships among these traits, and their associations with climate and
109 environmental variation, to investigate the factors responsible for shaping trait variation within and
110 between closely related species. To do so, we experimentally assayed physiological and pigmentation
111 variation in three population lines from each of three focal taxa within the *D. americana* species group—
112 *D. americana americana, D. americana texana*, and *D. novamexicana*, and combined these data with
113 bioclimate information from each species range and focal population. This approach allowed us to
114 disentangle the contributions of species-wide trait differences, local adaptation, and pleiotropic trait-
115 interactions to shaping the overall distribution of variation in these important physiological traits.
116

Methods

Experimental Fly stocks

Three stocks from each focal species, and one line from an outgroup species (*D. virilis*), were obtained from the University of California San Diego Drosophila Species Stock Center (DSSC). We used *Drosophila novamexicana* stocks from San Antonio, NM, Grand Junction, CO, and Moab, UT (15010-1031.08, 15010-1031.00, and 15010-1031.04 respectively); *D. americana americana* (hereafter *D. a. americana*) stocks from Chinook, MT, Chadron, NE, and White River, AR (15010-0951.02, 15010-0951.06, and 15010-0951.17 respectively); and, *D. americana texana* (hereafter *D. a. texana*) stocks from New Orleans, LA, Jamestown, SC, and Morrilton, AR (15010-1041.24, 15010-1041.29, and 15010-1041.23 respectively). The *D. virilis* stock originated from Texmelucan, Puebla, Mexico (15010-1051.48). *D. americana* is divided into subspecies according to presence of a chromosomal fusion of the X- and 4-chromosomes in *D. a. americana* that shows a distinct latitudinal cline (McAllister 2002). For simplicity we shall refer to them by their subspecies names. All fly stocks were reared on standard cornmeal media prepared by the Bloomington Drosophila Stock Center (BDSC) at Indiana University, and were kept at room temperature (~22C).

Environmental data

To quantify environmental variation in the natural range of our three focal species, we extracted bioclimatic variable data from documented collection locations of each species and used these data to create principal components that summarize the major axes of climate variation. First, geographical coordinate data were obtained for all known collections using TaxoDros (www.taxodros.uzh.ch) – a database that compiles latitude and longitude coordinates from published collection records. After curating for duplicates and erroneous or unspecified coordinates, we retained passport data for 10 *D. novamexicana*, 73 *D. a. americana*, and 68 *D. a. texana* population locations. For each of these

141 geographic locations, we extracted bioclimate variable data from two sources. From the Worldclim 2
142 database we extracted source location data at 30 arcsecond resolution for 19 bioclimatic variables (Fick
143 and Hijmans 2017); and, from the CliMond archive (Kriticos et al. 2012) we extracted 16 additional
144 bioclimatic variables at 10 minute resolution (see supplement). The latter were included despite this
145 relatively coarse resolution because they contained additional data on ultraviolet (UV) radiation and soil
146 moisture that were not available in the Worldclim registry. Many of these 35 bioclimatic variables
147 describe alternative aspects of temperature, precipitation, and seasonality over different time intervals,
148 so we performed a principal component analysis (PCA) in *R* on all 35 variables across all 151 population
149 locations to reduce non-independence and redundancy in this dataset. PCA uses orthogonal
150 transformation to generate a set of linearly uncorrelated principal components that summarize major
151 axes of variation across a dataset of potentially correlated variables. In our data, the first 3 PCs
152 explained 95% of the environmental variation across populations; variable loadings for these 3 PCs is
153 provided in the supplement (Table S1). Because the first 3 PCs explained ~85% of the variation across
154 populations, we used values for these PCs in our subsequent analyses of the relationship between
155 environmental variation and variation in physiological traits. Based on the variable loadings (Table S1)
156 we interpret the environmental significance of each of the first 3 PCs as follows: PC1 is strongly
157 negatively loaded for the majority of the precipitation and moisture index variables – with the exception
158 of the seasonality terms; therefore higher PC1 values indicate more arid areas with lower rainfall year-
159 round, and higher daily and seasonal temperature variability. PC2 is most strongly loaded for radiation
160 and temperature extremes for the positive values, and negatively loaded for ground moisture variables
161 and temperature/UV seasonality. We therefore interpret high values of PC2 to indicate year-round high
162 temperature and radiation, with low moisture, and negative values to indicate areas with year-round
163 high moisture along with high seasonality in temperature and radiation. Last, negative PC3 values
164 indicate locations where the hottest and wettest periods of year align, and are accompanied by high

165 radiation and moisture, while positive PC3 values indicate high temperatures and radiation during the
166 driest times of year when precipitation is low.
167
168 *Desiccation resistance assay*
169 To assess population, species, and sex specific differences, desiccation resistance was assayed in
170 replicate for individual males and females of each of our nine focal populations, using custom
171 desiccation vials. Virgin flies were isolated within 24 hours of eclosion and aged individually for 3 days
172 prior to the start of the experiment. Flies were then mouth aspirated individually into a modified
173 *Drosophila* culture vial which contained a layer of 20g of Drierite, 10g of desiccated cork, and a piece of
174 cheesecloth, and was sealed by a layer of parafilm. Each fly was placed above the cheese cloth and cork
175 (in order to avoid direct contact with Drierite that negatively effects survival) and observed every 15
176 minutes until death. Desiccation resistance was then quantified as the total time in minutes that each
177 individual survived in a desiccation vial. A minimum of 5 replicates were performed per sex for each
178 population. Trials were performed in blocks in which one fly of every identity (population x sex) was
179 assayed simultaneously, to avoid confounding sex or population effects with trial date. At the end of the
180 survival assay, each individual was weighed to obtain their dry weight (as a proxy for size) to include as a
181 covariate in survival analyses. Dry (post-death) weight was determined to be an effective proxy for wet
182 (pre-desiccation) weight in a pilot experiment in which individuals of each population and sex were
183 weighed before and after individual desiccation trials (Pearson's correlation, females: $r(8) = 0.899$, $P <$
184 0.001; males: $r(8) = 0.925$, $P < 0.001$).
185
186 *UV irradiation resistance assay*
187 We assessed UV-B resistance for each sex within each population (including the *D. virilis* line), at
188 each of four different exposure intensities: 100, 500, 1000, and 5000 Joules/$m^{2,}$ plus a control assay at 0

189 J/m^2. UV resistance trials were performed similarly to Matute and Harris (2013) and Aguilar-Fuentes et
190 al. (2008). Briefly, virgin males and females of each population were isolated and kept in single-sex
191 groups of 20 for 24 hours prior to experiment start. Each group of 20 flies was then lightly anesthetized
192 and weighed as a group before being irradiated with UV-B light at one of the four experimental
193 intensities using an ultraviolet Stratalinker 2000 (Stratagene, La Jolla, CA). For the 0J exposure - which
194 essentially measures longevity in the absence of acute UV exposure - flies were simply anesthetized,
195 weighed, and placed in the Stratalinker without UV exposure. Each group was then transferred to a vial
196 containing standard cornmeal media and scored once daily for number of flies still alive; groups were
197 transferred to fresh food vials as often as necessary. The experiment continued until all flies in each vial
198 were dead. For each assayed energy level, trials for both sexes in all ten lines were initiated
199 simultaneously, to avoid confounding these factors with date effects.
200
201 *Pigmentation assay*
202 Dorsal abdominal pigmentation was assessed on individual males and females from each focal
203 population (and for the *D. virilis* line), in a similar manner to Wittkopp et al. (2010, dataset 'A'). Briefly,
204 individual 7-day old virgin flies for each sex and population were placed in 10:1 ethanol to glycerol
205 mixture and stored at room temperature for 1-5 days. The dorsal abdominal cuticle (specifically tergites
206 A3-A5) was dissected from each fly, isolated from other tissues, and mounted in Hoyer's solution. Each
207 cuticle was then viewed and digitally imaged on an AMG EVOS FL scope (AMG, Bothell, WA, USA) under
208 standardized light conditions. Body color was quantified on gray-scale images of each cuticle by
209 calculating the average median pixel intensity of 20 randomly-selected, non-overlapping regions on a 0-
210 255 scale (avoiding the dorsal midline which has consistently lighter pigmentation), in Image J (NIH,
211 Bethesda, MD, USA). Five replicate individuals from each sex within each population were assessed.
212

Statistical analyses

Trait differences between sex, species, and populations

All statistical analyses were performed in R version 3.4.3, as was figure construction. To first assess the distribution of variation in our traits, we analyzed each physiological trait for differences between species, populations, and sex. For each of desiccation resistance and pigmentation, we fit a multi-way ANOVA with sex, species, and population nested within species, as independent variables, and each trait as the response variable. For desiccation resistance, dry weight was also included as a fixed effect to account for individual body size. For both of these traits we also performed post-hoc contrasts between each pair of species using the Tukey test.

For UV resistance, effects of sex, species, and population were assessed differently because the data for each group within each treatment are best summarized and compared as Meier-Kaplan survival curves. A cox proportional hazard model (Therneau 2013) was first used to determine the effect of sex between all survival curves across treatment levels and species, and sexes were found to differ (Wald test (1) = 105.2, $P < 0.0001$). Survival also consistently differed between sexes within each treatment level (see supplement), and all subsequent analyses were performed separately on each sex. To assess species and population effects on UV resistance within each of the five different treatment levels, two cox proportional hazard models were constructed: one to test for significant effects of species (where populations were grouped by species) and the other to test for significant differences between populations irrespective of species. The Wald test was used to evaluate the overall effects of terms included in each model. Finally, we used the median of the Meier-Kaplan curve estimate—equivalent to the day in which 50% of the flies in a given trial are dead—as a summary statistic for UV resistance for each sex in each population at each given treatment level, for use in subsequent analyses of trait-trait associations and trait associations with environmental PC axes (see below).

Environmental variation and association with physiological traits

We tested for evidence that species significantly differed in environment from one another by performing one-way ANOVAs with species as the independent variable and each of the first three PC axes as the dependent variables. These analyses were performed with data from all collection localities used to generate the PC axes (N=149), and also on the set of nine focal populations used for our trait analyses. For each analysis with a significant species effect, we also performed Tukey post-hoc contrasts to determine which species differ from one another on each PC axis.

To look for evidence of local adaptation (specifically trait-environment associations), for each sex separately we evaluated the relationship between population physiological trait means (or, for UV resistance, median survival values) and population values for principal component axes of environmental variation, while taking into account broader environmental differences between species. Because our PC axes showed statistical separation among species—that is, *D. novamexicana* had complete separation from the other two taxa along both PC1 and PC2 axes (Figure 3)—for each PC we first calculated the residuals from a one-way ANOVA with species as the independent variable, and then used these residual PC values in our analyses of population-level effects. Then, for each of the first three PCs separately, we fit an ANOVA with residual PC values and species as independent variables, and the mean population trait value for either desiccation resistance or pigmentation as the response variable. A similar set of models were performed with UV resistance data from each UV treatment level, but median Meier-Kaplan curve estimates (as described above) as the response variable. These analyses allowed us to simultaneously evaluate the contribution of both species differences and local environmental variation to variation in each physiological trait, and therefore assess how each PC contributes to variation in a given trait within each species. Because we performed 6 tests for each trait, the Bonferroni-corrected significance level is p=0.008 for each trait.

Finally, we examined the strength of pairwise associations between each of our phenotypic traits of interest (desiccation resistance, pigmentation, and UV resistance at each of five levels), using Pearson's correlation coefficients. Analyses were performed using population means (because each trait was measured on different individuals and, for UV resistance, groups of individuals), and on each sex separately (as there was evidence that each trait is moderately to strongly different between sexes; see above and results).

Results

Species differed along axes of environmental variation

Across all species collection records (N = 151), we found that species differ along PC1 ($F(2, 82.2)$, $P < 0.001$) and PC2 ($F(2, 59.76)$, $P < 0.001$), but not PC3 ($F(2, 3.4)$, $P = 0.065$), environmental axes. Post-hoc Tukey tests indicated that all 3 species differed from one another for both PC1 and PC2 (all P values < 0.001). Despite modest power, species comparisons using environmental data from only our nine focal populations were similar: Species differed along PC1 ($F(2, 17.4)$, $P = 0.003$) and PC2 ($F(2, 6.6)$, $P = 0.030$), but not PC3 ($F(2, 0.7)$, $P = 0.550$). For PC1, *D. novamexicana* differed from both *D. a. americana* ($P = 0.033$) and *D. a. texana* ($P = 0.003$), although the two *americana* subspecies did not differ ($P = 0.100$); for PC2, *D. novamexicana* differed from *D. a. americana* ($P = 0.026$), but the other two contrasts (*D.nov – D. tex:* $P = 0.147$; *D.am – D.tex:* $P = 0.400$) were not significant.

Traits differed among both species and populations and, in some cases, sexes

Abdominal pigmentation differed between species ($F(2, 11.9)$, $P < 0.001$) and between populations within species ($F(6, 3.1)$, $P = 0.008$), and marginally differed between sexes ($F(1, 0.06)$, $P = 0.063$). *D. novamexicana* were the lightest flies, and post-hoc Tukey tests confirmed *D. novamexicana* was significantly less pigmented than *D. a. americana* ($P < 0.001$) or *D. a. texana* ($P < 0.001$), while the

284 two americana subspecies had similar pigmentation (P = 0.96) (Figure 2). With respect to sex-specific
285 differences, females were marginally less pigmented than males.

286 Desiccation resistance also differed between species (F(2, 29.8); P < 0.001) as well as between
287 populations within species (F(6, 4.5); P < 0.001), but not between sexes (F(1, 0.8); P = 0.364); dry weight
288 (body size) had no effect (F(1, 1.3), P = 0.25). Post-hoc Tukey tests indicated significant pairwise
289 differences between all three species (*D.nov – D. am:* P < 0.001, *D.nov – D. tex*: P < 0.001; *D.am – D. tex:*
290 P = 0.002); *D. novamexicana* had the greatest desiccation resistance, followed by *D. a. texana* and *D. a.*
291 *americana*. Consistent with this, *D. novamexicana* populations generally performed better than the
292 other populations, with the exception of *D. a. texana* from Morrilton, Arkansas, which had the third
293 highest survival overall (Figure 3).

294 Species and population differences for UV resistance were assessed within each treatment and
295 for each sex separately (see methods and supplement for sex-specific analyses). For females, species
296 and populations differed in survivorship after 0J, 100J, 500J, and 5000J of UV exposure, but not after
297 1000J (Table 1). For males we found a significant species effect on survival after 0J and 100J of UV
298 exposure, but not after 500J, 1000J, or 5000J; results for population-level analyses were the same
299 except UV exposure to 1000J was also significant (Table 1). Despite these significant differences, the
300 rank order of UV resistance between populations varied idiosyncratically among different UV exposure
301 levels, and was generally inconsistent between them (Figure S1).

302

303 *Pigmentation shows different patterns of environmental association within- and between-species, while*
304 *desiccation resistance displays predominantly species-level patterns.*

305 In addition to large significant differences in pigmentation among species for both sexes, we
306 found that intraspecific pigmentation variation was modestly associated with PC2 for both males and
307 females, although not with PC1 or PC3 (Table 2) (Figure 3). Based on the factor loadings for PC2 (see

methods and Table S1), this latter finding indicates that higher year-round UV exposure and temperature coupled with low moisture (i.e. positive values of PC2), are associated with relatively darker pigmentation within each of these species. This association is particularly interesting as the broader-scale differences between species show the opposite association, where the species with the lightest pigmentation—*D. novamexicana*—is clearly associated with positive values for this environmental axis (Figure 3).

In contrast to pigmentation, for all three PCs we found that species differences were modestly explanatory of desiccation resistance variation among males (but not females) (Table 2), whereas intraspecific resistance variation was not associated with residuals of any of the three PCs for either sex (Table 2) (Figure 4). Based on these results, differential desiccation resistance in males is generally consistent with large-scale environmental differences between species (in particular, *D. novamexicana* males are more desiccation resistant), but there is little evidence for additional local adaptive variation within species for either sex for this trait. For UV resistance, associations with environment were assessed within each treatment level and sex, but we found only one significant association after correcting for multiple testing: for the 5000J treatment, females showed a significant association with PC2 ($F(1) = 12.75$; $P = 0.009$). Full results of these analyses are found in the supplemental material (Table S3).

For associations between traits, we found that lighter pigmentation is positively correlated with higher desiccation resistance in males ($r(7) = 0.70$, $P = 0.036$), but only marginally for females ($r(7) = 0.59$, $P = 0.091$)(Figure S2). In contrast, UV resistance was not correlated with pigmentation for either sex at any of the treatment levels (Table S4). UV resistance was modestly negatively correlated with desiccation resistance for females at the highest exposure (5000J, $r(7) = -0.720$, $P = 0.029$), but this association does not survive multiple-testing correction.

Discussion

Here we surveyed natural variation in three physiological traits in the *Drosophila americana* species group, examined how environmental factors have shaped this variation, and how these traits might influence each other. We found strong differences between species in their environmental associations, as well as in pigmentation and desiccation resistance variation, but not in assayed UV resistance. When we evaluated evidence of adaptation by examining trait-environment associations, we found evidence for a complex role of natural selection in shaping pigmentation variation; within-species variation suggests that darker flies occur in consistently hotter, drier, higher radiation environments, but between-species the opposite pattern is evident. Conversely, desiccation resistance was largely differentiated by species-level patterns, and UV resistance phenotypes showed no clear association with measured environmental variation or these other traits. These different patterns of association between environmental and trait variation, both within and between species, suggests that the temporal and spatial scale at which selection acts on these traits is different, as are the major selective drivers responsible for patterns of observed variation.

Pigmentation variation is shaped by alternative factors within and between species

The observation that *Drosophila* species, sexes, and/or populations vary in pigment is common (see Introduction) and has prompted several, sometimes conflicting, hypotheses about the functional role of this variation. Many of these hypotheses are based on the general observation that pigmentation is associated with spatial variation, especially latitudinal gradients: Previous analyses along latitudinal clines in *Drosophila* have shown evidence for both increasing (David et al. 1985, Munjal et al. 1997, Telonis-Scott et al. 2011, Capy et al. 1988, Heed and Krishnamurthy 1959) and decreasing (Bastide et al. 2014) melanization with latitude. Here, one of our goals was to more directly evaluate this trait variation

355 by associating pigmentation with axes of bioclimatic variation (rather than solely geographic patterns),
356 and with other physiological trait variation, to assess the plausibility of these prior hypotheses in our
357 focal *Drosophila* species. We found both that our significantly less pigmented species—*D.*
358 *novamexicana*—is found in consistently hotter, drier, higher radiation environments, and also that
359 *darker* pigmentation is moderately positively associated with these same environmental factors within
360 each species. Our observations suggest that environment variation imposes different directions of
361 selection on this trait at the species level versus the local level.

362 These results also provide new data with which to evaluate three alternative hypotheses
363 proposed to explain natural variation in pigmentation found in other *Drosophila* species. The first of
364 these hypotheses—the thermal melanism hypothesis (or Bogert's Rule in ectotherms)—proposes that
365 more pigment allows better absorption of thermal radiation in colder environments. This proposal has
366 been commonly invoked to explain a geographical association between darker pigmented ectotherms
367 and higher latitudes (Gaston et al. 2009). In our data, at the species level there is some evidence that
368 flies in colder regions are darker, at least when comparing *D. a. americana* to *D. novamexicana*.
369 However, several other aspects of our data are inconsistent with the association between pigmentation
370 and colder environments proposed by the thermal melanism hypothesis: *D. a. americana* and *D. a.*
371 *texana* do not significantly differ in pigmentation, even though *D. a. texana* is both from lower latitudes
372 and regions with warmer temperatures. Furthermore, at the population level within all three species we
373 see the opposite association, where darker flies are associated with more consistently warm and higher
374 radiation areas.

375 A second hypothesis proposed to explain pigmentation variation is that it plays a direct role in
376 UV protection. Bastide et al. (2014) showed in Sub-Saharan African populations of *D. melanogaster* that
377 darker morphs are found at lower latitudes, and that UV exposure (among other environmental factors)
378 was the factor most highly correlated with pigmentation variation, from which they inferred that there

379 is strong evidence that pigmentation plays a role in UV protection (Bastide et al. 2014). Within each
380 species examined here, our data are consistent with Bastide et al.'s observations, as populations from
381 areas with higher year-round UV radiation and temperatures (PC2) have darker pigmentation. However,
382 we found little evidence for strong associations between UV resistance *per se* (as measured by our
383 assay) and either pigmentation or environment (as discussed below), so the exact causal relationship
384 between UV resistance and pigmentation in our study remains uncertain. It has been previously shown
385 in *D. melanogaster* that *ebony* mutants (which are more darkly pigmented) live significantly longer than
386 lighter pigmented *yellow* mutants when both are exposed to UV irradiation (Wang et al 2008), giving
387 some credence to the proposal that pigmentation could be important for UV resistance. However, even
388 if selection imposed by UV stress does indeed shape some of the pigmentation variation within
389 species—specifically by increasing pigmentation in high UV environments—this cannot explain the
390 pattern of pigmentation we observe *between* species where the least pigmented species is found in
391 drier, more consistently hot environments with greater UV exposure (high PC2). This apparent
392 inconsistency between trait-environment associations at alternative evolutionary scales (within and
393 between species) itself indicates that UV resistance cannot be a complete explanation of the
394 pigmentation variation we observe here. Instead, additional patterns of selection and/or demographic
395 history appear to be responsible for species-level differences—particularly the lighter pigmentation of *D.*
396 *novamexicana*—although the nature of these forces and when they acted are harder to determine.
397 Wittkopp et al. (2009) showed that the yellow body color of *D. novamexicana* is a fixed, derived
398 phenotype determined by alleles at the *tan* and *ebony* loci, and inferred that these alleles pre-date
399 speciation, as they can also be found segregating across populations of *D. americana*. The broader
400 distribution of these shared pigmentation-variation alleles suggests that historical selection could have
401 played a role in shaping genetic variation prior to speciation, although it doesn't indicate the specific
402 selective agent(s) responsible. Moreover, despite these shared alleles, contemporary *D. novamexicana*

403 lineages are clearly significantly less pigmented than even the lightest *D. americana* populations. This
404 suggests that additional species-specific forces have acted since the split of their common ancestors,
405 that might include the fixation of light alleles via drift, or the influence of other species-wide selective
406 agents. For example, although evidence in *Drosophila* is currently lacking (Wittkopp and Beldade 2009),
407 prior studies indicate that pigmentation can play a role in sexual signaling in other insects (True 2003).
408 Therefore, exaggerated pigmentation differences between species could result from pleiotropic effects
409 on mating preferences within and between species, a hypothesis that is testable with future work.
410 Regardless of the actual cause of species pigmentation differentiation, at present our data suggest that
411 different selective pressures operating at different times and scales could produce complex, and even
412 opposing, patterns of environmental association with a single trait, especially when the trait has more
413 than one biological function.

414 Aside from the above proposals, a third class of hypotheses about the evolution of pigmentation
415 focuses on its proposed association with desiccation resistance. One prevailing hypothesis is that
416 increased melanization confers thicker cuticles that increase tolerance to desiccation (Parkash et al.
417 2008), and increased melanism is strongly correlated with differences in desiccation resistance in some
418 *Drosophila* (Brisson et al. 2005, Rajpurohit et al. 2008, Karan et al. 1998, Parkash et al. 2008). While we
419 did find a modest correlation between desiccation resistance and pigmentation, the relationship we
420 detected is in the opposite direction than is proposed under this hypothesis. Furthermore, genetic
421 analysis in this group indicates little evidence that these traits are mechanistically associated. In
422 particular, Wittkopp et al. 2010 used *D. novamexicana* and *D. americana* to create backcrossed lines
423 with *ebony* or *tan* alleles introgressed from the opposite species; they found that introgression of darker
424 or lighter alleles into the background of the opposing species did not alter desiccation resistance,
425 providing strong evidence that these traits are not mechanistically associated, at least at these large
426 effect loci. An alternative to a direct mechanistic connection is that these trait associations could simply

427 emerge due to their shared covariation with other, causal, axes of environmental variance. This
428 possibility highlights the need for more studies that simultaneously address these associations between
429 multiple traits, species, and environmental gradients, in order to better disentangle alternative
430 explanations of the forces shaping complex patterns of trait variation.

431

432 *Desiccation resistance is shaped by factors acting among species rather than at local spatial scales*

433 Unlike pigmentation variation, the adaptive connection between species trait differences and
434 the overall climate of their natural ranges is more readily understood for desiccation resistance: *D.*
435 *novamexicana* is from the xeric regions of the US and has significantly elevated desiccation resistance
436 relative to its mesic sister species, presumably because this confers improved survivorship throughout a
437 substantially drier species range. It is intriguing, then, that there is no consistent relationship between
438 desiccation resistance and any PC axis within each of our species. As temperature and UV exposure
439 become increasingly and consistently high (as shown by increasingly positive values of PC2) desiccation
440 resistance generally increases across our focal populations (Figure 4); however, species differences
441 clearly determine this pattern (Table 2), with little evidence for additional within-species local
442 adaptation in response to this axis. This lack of a stronger association within species could be due to
443 several non-mutually exclusive factors, although in the absence of more direct data they remain
444 hypotheses. First, our assay only assesses one element of physiological tolerance to desiccation,
445 whereas there are multiple ways to avoid desiccation stress in the wild. Populations within our species
446 might therefore be responding to local variation in desiccation stress via strategies or behaviors that are
447 not observable in the experimental assay we used here. A similar proposal was suggested by Sillero et al.
448 (2014) to explain a lack of association between source environment and thermal performance in these
449 same species. Second, while bioclimatic variables can be very effective in describing macro-level

450 associations between populations and environment, they do not provide a complete view of microscale
451 differences in abiotic and biotic environments. Consequently, small scale environmental differences,
452 such as shade availability or proximity to local water sources, will be unaccounted for in these analyses
453 even though these might be important elements shaping trait-environment relationships. Finally, there
454 could be additional within-species constraints on the mechanisms underpinning desiccation resistance,
455 such that they are either unable to respond more finely to local conditions, or the fitness benefits of
456 doing so are insufficient to outweigh the benefits of optimizing alternative functions. This is especially
457 relevant for traits that can serve several different functional roles (i.e. are pleiotropic), and that
458 experience trade-offs between these functions. One such trait that could be relevant to desiccation is
459 cuticular hydrocarbon (CHC) composition—the blend of waxy compounds on the surface of insects—
460 that is known to be important for desiccation resistance in multiple species, but can also play a role in
461 sexual communication (reviewed Chung and Carroll 2015). For example, if maintaining effective species-
462 wide CHC-mediated sexual signaling imposes constraints on local physiological responses in CHC-
463 mediated desiccation resistance, this could lead to a pattern in which species strongly differ in
464 desiccation resistance (in a way that matches broad environmental variation), but there is no local
465 pattern of adaptation in the same physiological response. In this way, when responses to a general
466 physiological stress can be achieved by an amalgam of alternative adaptive 'solutions', some of which
467 are subject to additional functional constraints that differ between evolutionary and geographical scales,
468 observed patterns of variation in individual traits need not fully reflect the complexity of these adaptive
469 trait responses.

470

471 *Life history differences between sexes, populations, and species might have additional ecological*
472 *functions*

While our results for pigmentation and desiccation resistance reveal differences at the population and/or species level that are consistent with selective responses to abiotic environmental variation, the patterns that emerge from our UV resistance assay are less clear. Although we detected population and species differences in some individual treatments, these results were generally weak and idiosyncratic with respect to their direction and magnitude. This might reflect the limitations of extrapolating natural functional differences in UV tolerance from survivorship after an acute single exposure to UV (a technique that is commonly used; Matute and Harris 2013, Jacobs 1974, Wang et al. 2008). Exposing flies to moderate to high doses of UV radiation in a single exposure may not be analogous to UV resistance to low levels of radiation on a daily basis; more realistic assays, with periodic or sustained exposures of lower average dose, might better simulate differences in natural environmental conditions. Regardless, this assay did reveal at least one interesting life-history difference among our groups: a consistent pattern of significantly increased longevity in females relative to males, regardless of species, as well as longevity differences among some of our populations. Because these differences were also apparent in the control (0J) exposure, it is likely that they largely reflect baseline longevity differences between these groups. Our finding is generally consistent with a previous analysis of longevity in this species group that also found that females tended to live longer than males, at least in some strains (Durbin and Yoon 1986). Greater female longevity is a pattern seen across many, but not all, *Drosophila* species (Yoon et al. 1990), and several factors could explain both sex and strain differences in lifespan, including sex-linkage of the underlying causal factors but also life history differences associated with ecological variation. The lifespan variation we observe here might therefore provide another opportunity to assess the ecological significance of additional quantitative trait variation, in the future.

Conclusions

Our study took a multi-faceted approach to assessing the factors responsible for natural variation in a set of ecologically-relevant physiological traits. By simultaneously assaying variation both within and between species, we were better able to discern how geospatial variation can influence patterns of trait variation in ways that can be complex. Our finding that pigmentation-environment associations differ between evolutionary scales indicates that pigmentation variation is shaped by different factors within and between species. The contrasting environment associations found for our other two traits—few clear associations for assayed UV resistance, but species-level differentiation in acute desiccation resistance that matched species-wide differences in natural climate conditions--indicate that while pigmentation, desiccation resistance, and UV resistance frequently appear to covary, this pattern need not indicate that these traits are directly mechanistically connected. Together, the specific nature of trait and environment associations observed at each scale provided new data to assess several conflicting explanations previously proposed for pigmentation variation in *Drosophila*, including whether pleiotropy plays an important role in determining the distribution of this natural variation. The multilevel approach that we have taken here can therefore potentially provide more nuanced insights into complex relationships among traits, and between traits and environmental variation, that both suggest resolutions to apparently contradictory patterns in the literature and generate additional hypotheses for future mechanistic and ecological study.

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613 **Table 1: Population differences in UV survivorship within treatment levels. Survivorship curves were**
614 **constructed for each treatment according to a cox proportional hazard model, and statistical**
615 **differences between populations within treatments was assessed using the Wald test. 9 degrees of**
616 **freedom for each analysis. Bonferroni corrected significance level is *P* < 0.005.**
617

Energy	**Male**		**Female**	
	F value	*P* value	*F* value	*P* value
0J	32.42	0.0001	62.88	< 0.0001
100J	31.35	0.0003	72.26	< 0.0001
500J	8.68	0.4669	41.2	< 0.0001
1000J	36.98	< 0.0001	16.9	0.0503
5000J	9.74	0.3719	33.42	0.0001

618

619

Table 2: Associations between principal component (PC) axes of environmental variation and physiological traits for these species. Reported statistics are from multi-way ANOVA models that include a PC (d.f.=1) and species (d.f.=2) as independent variables, and a given physiological trait as the dependent variable. Bonferroni corrected significance level is *P* < 0.005.

	Desiccation Resistance				**Pigmentation**			
	Male		*Female*		*Male*		*Female*	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
PC1	0.89	0.39	0.34	0.58	1.17	0.33	1.12	0.34
Species	7.08	0.035	2.61	0.17	46.43	0.00059	81.05	0.00016
PC2	0.20	0.67	0.33	0.58	7.28	0.043	11.56	0.019
Species	6.25	0.044	2.61	0.17	92.42	0.00011	219.13	<0.0001
PC3	3.64	0.12	3.84	0.11	2.65	0.16	2.95	0.15
Species	10.38	0.017	4.31	0.082	57.60	0.00035	105.25	<0.0001

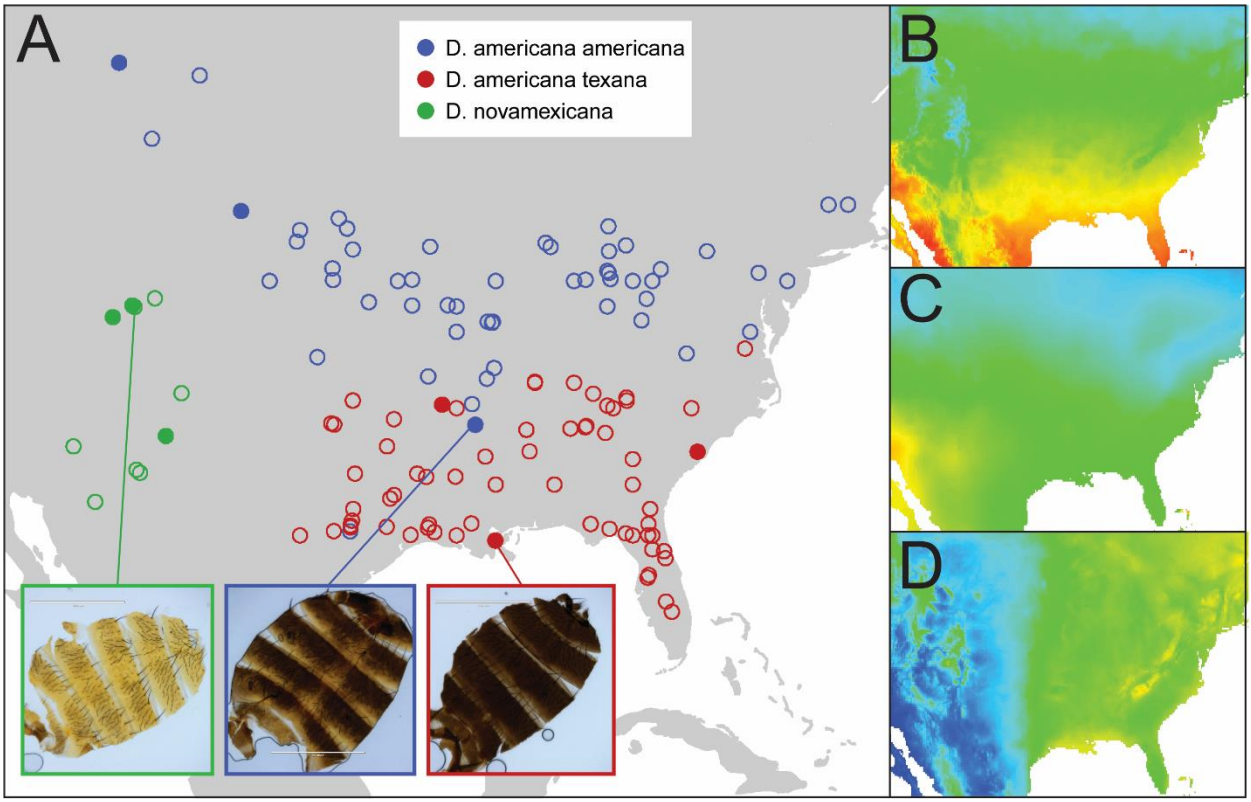
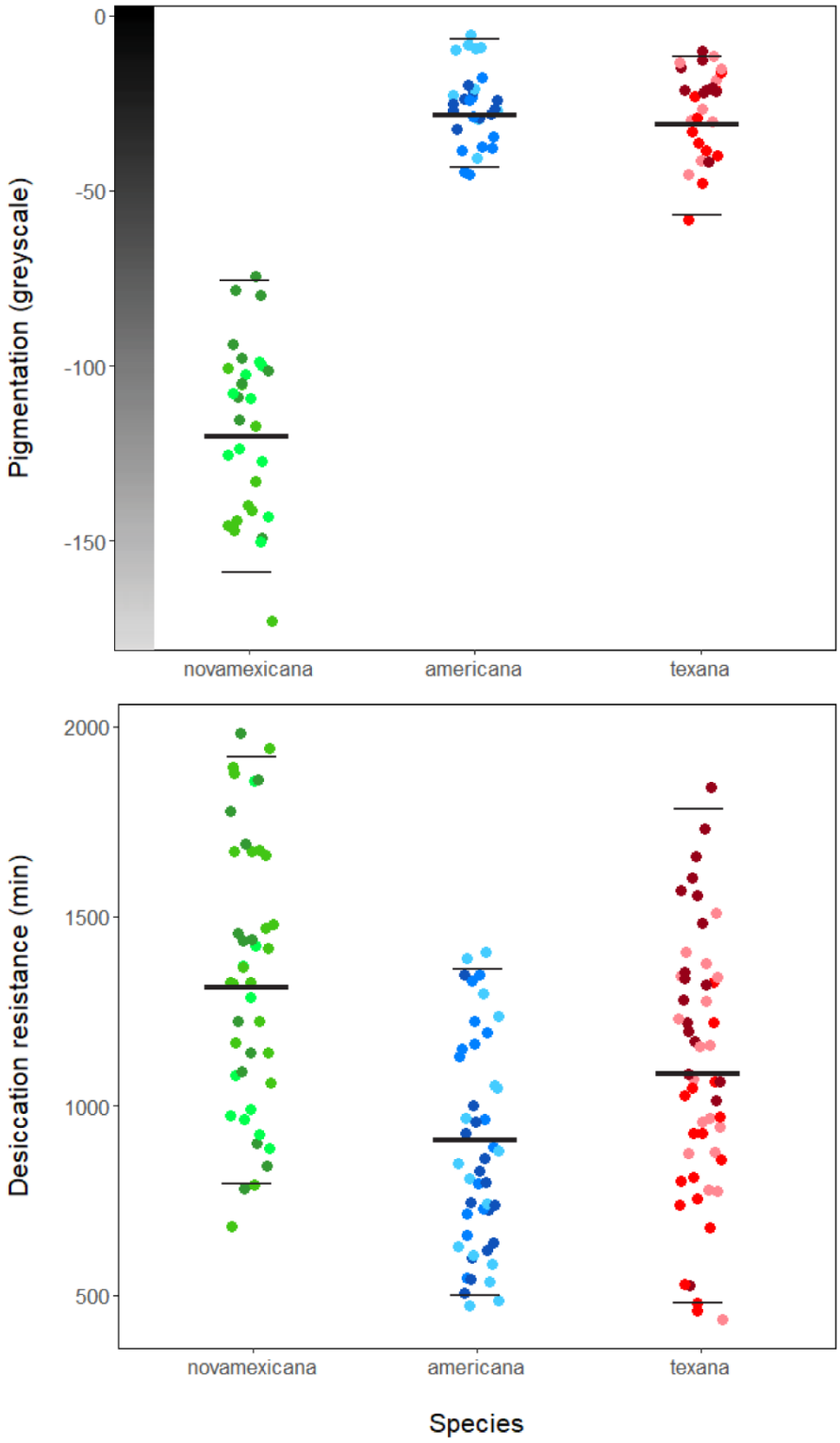


Figure 1: Distribution of collection locations and major environmental variables for our three focal species. Panel A shows collection records for each of *D. novamexicana* (green), *D. a. americana* (blue), and *D. a. texana* (red) as obtained from the TaxoDros database (taxodros.uzh.ch, see methods). Closed circles indicate the nine sample locations for populations used in this study. Panel B-D show heatmaps of spatial variation in annual mean temperature (B), annual mean radiation (C), and annual mean moisture index (D), as obtained from the Worldclim and Climond databases. Cuticle dissection images inset into Panel A are representative male cuticles from (left to right) Grand Junction, Colorado, White River, Arkansas, and New Orleans, Louisiana.



642

643 **Figure 2: Distribution of individual values for pigmentation (top) and desiccation resistance (bottom,**
644 **in minutes), according to species (X axis) and population within species (shade variation among**
645 **points). The y-axis values for individual cuticles on the pigmentation graph correspond to a computed**
646 **greyscale value represented by the gradient bar. The thick bars indicate species means; thin bars**

indicate 95% confidence intervals around the mean. Pigmentation differs between both species (*F*(2, 11.86), *P* < 0.0001) and populations within species (*F*(6, 3.13), *P* = 0.0083). Post-hoc contrasts indicate *D. novamexicana* is significantly lighter than *D. a. americana* (*P* < 0.0001) and *D. a. texana* (*P* < 0.0001); *D. a. americana* and *D. a. texana* do not differ (*P* = 0.96). Desiccation resistance differs between species (*F*(2, 29.76); *P* < 0.0001) and populations within species (*F*(6, 4.48); *P* = 0.0004). Post-hoc tests indicate significant differences in all pairwise contrasts (*D.nov – D. am: P* < 0.0001, *D.nov – D. tex: P* = 0.00039; *D.am – D. tex: P* = 0.0023).

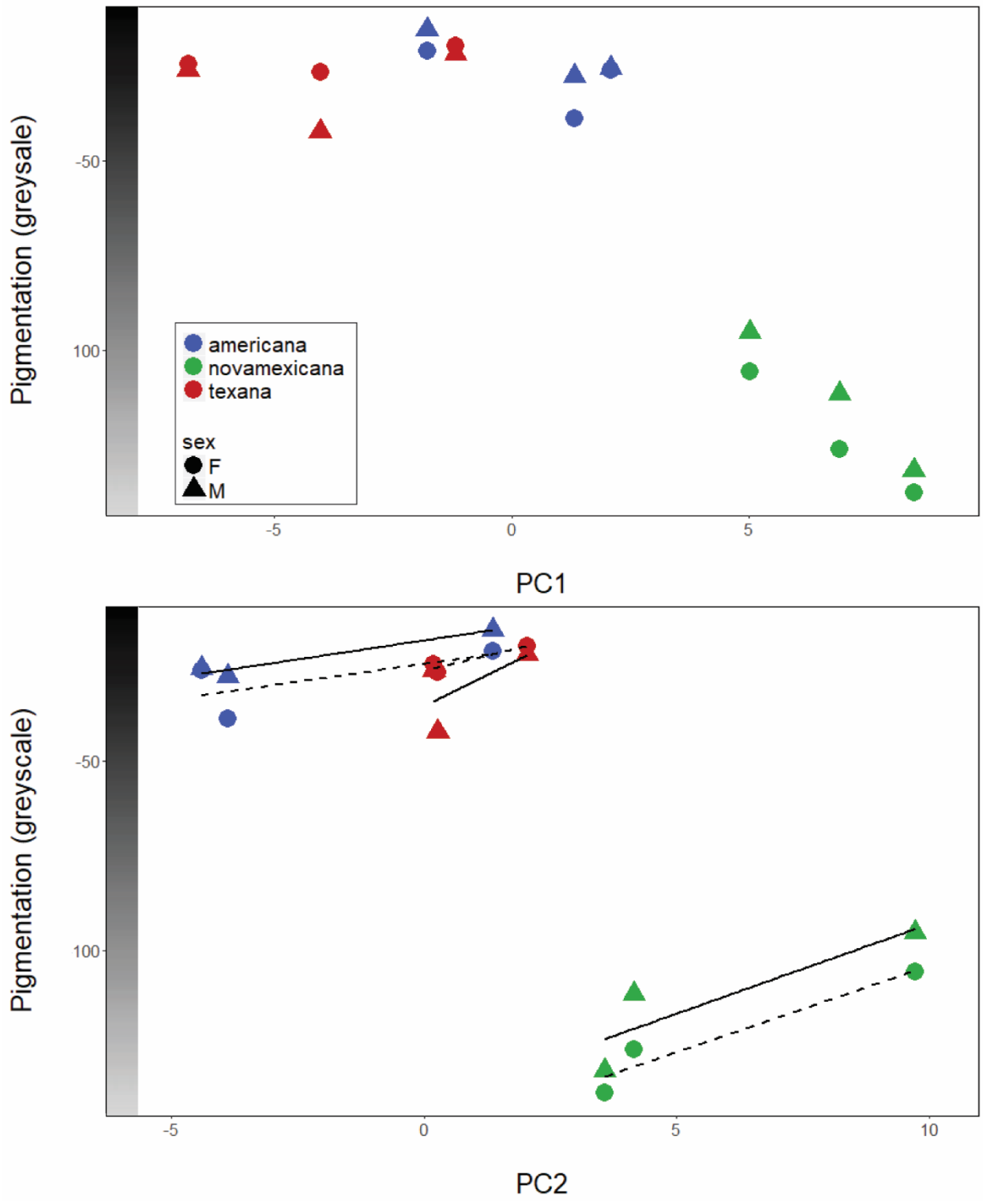


Figure 3: Relationship between mean male (triangle) and female (circle) pigmentation in each population and PC1 (top panel) or PC2 (bottom panel). The y-axis values on the pigmentation graph

correspond to a greyscale value represented by the gradient bar. Pigmentation was not associated with PC1 (annual rainfall and moisture) for either males (*F*(1) = 1.17; *P* = 0.33) or females (*F*(1) = 1.12; *P* = 0.34) when also accounting for species differences. Pigmentation was modestly associated with year-round high temperature and radiation (PC2) for both males (*F*(1) = 7.28; *P* = 0.043; solid lines) and females (*F*(1) = 11.56; *P* = 0.019; dashed lines) when also accounting for species differences (Table 2).

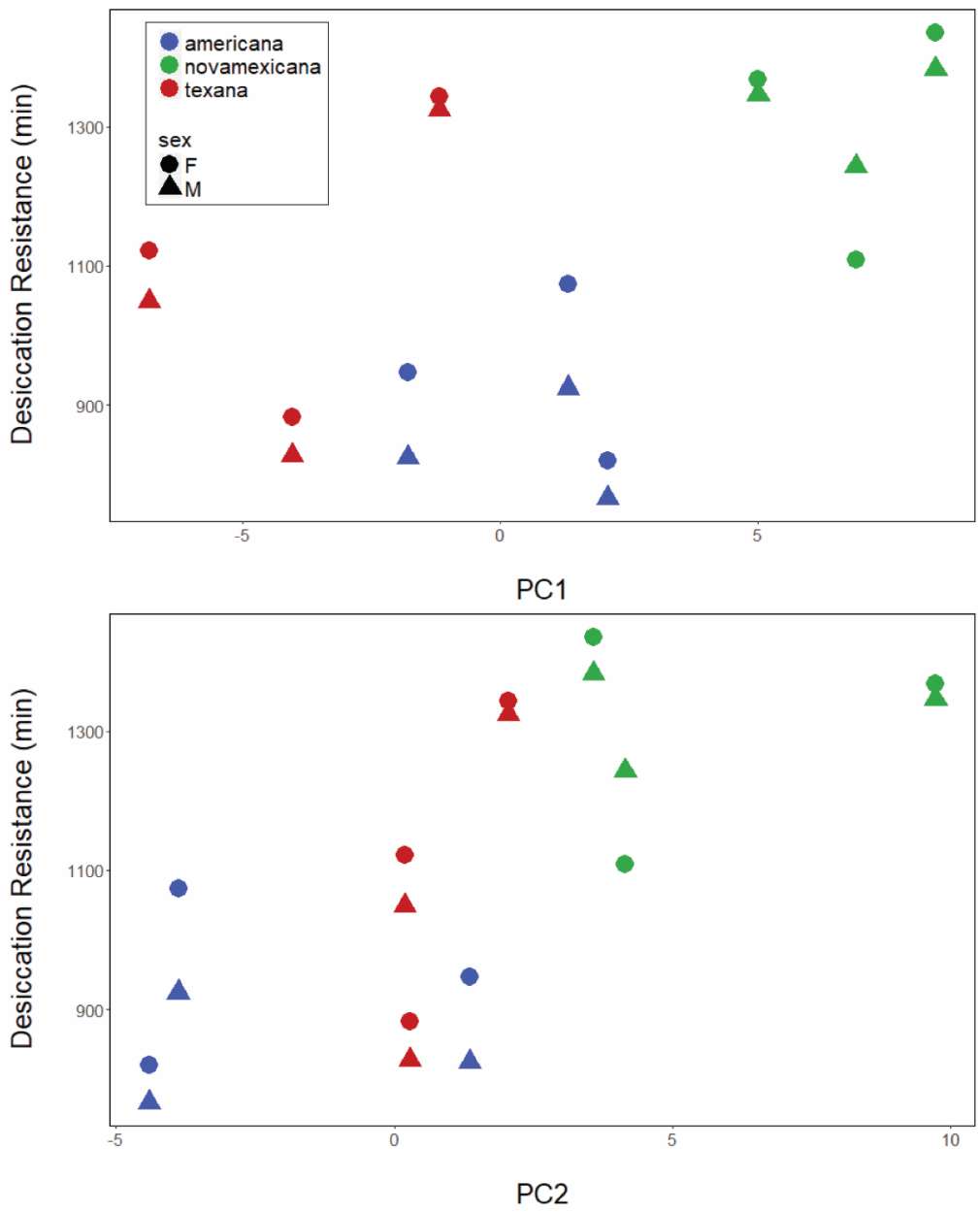


Figure 4: Relationship between population mean desiccation resistance and PC1 (top) and PC2 (bottom). Desiccation resistance was not associated with PC1 (annual rainfall and moisture) or PC2, for either sex, when also accounting for species differences (see Table 2). In comparison there was a modest species effect when analyzing associations between desiccation resistance and PC1($F(2) = 7.08$; $P = 0.035$) , and with PC2 ($F(2) = 6.25$; $P = 0.044$) (Table 2).