

ORIGINAL ARTICLE

Local adaptation for body color in *Drosophila americana*PJ Wittkopp^{1,2}, G Smith-Winberry¹, LL Arnold², EM Thompson², AM Cooley¹, DC Yuan², Q Song² and BF McAllister³¹Department of Ecology and Evolutionary Biology, Ann Arbor, MI, USA; ²Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI, USA and ³Department of Biology, University of Iowa, Iowa City, IA, USA

Pigmentation is one of the most variable traits within and between *Drosophila* species. Much of this diversity appears to be adaptive, with environmental factors often invoked as selective forces. Here, we describe the geographic structure of pigmentation in *Drosophila americana* and evaluate the hypothesis that it is a locally adapted trait. Body pigmentation was quantified using digital images and spectrometry in up to 10 flies from each of 93 isofemale lines collected from 17 locations across the United States and found to correlate most strongly with longitude. Sequence variation at putatively neutral loci showed no evidence of population structure and was inconsistent with an isolation-by-distance model, suggesting that the pigmentation cline exists despite extensive gene

flow throughout the species range, and is most likely the product of natural selection. In all other *Drosophila* species examined to date, dark pigmentation is associated with arid habitats; however, in *D. americana*, the darkest flies were collected from the most humid regions. To investigate this relationship further, we examined desiccation resistance attributable to an allele that darkens pigmentation in *D. americana*. We found no significant effect of pigmentation on desiccation resistance in this experiment, suggesting that pigmentation and desiccation resistance are not unequivocally linked in all *Drosophila* species.

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Introduction

Clinal variation, in which the average value of a trait changes gradually over a geographic area, can be caused by either neutral or non-neutral evolutionary processes (reviewed by Kawecki and Ebert, 2004). For example, the neutral process of genetic drift can generate a cline through spurious correlations between physical locations and segregating polymorphisms. Limited migration between populations (especially when migration rates are correlated with geographic distance) promotes cline formation. Alternatively, natural selection can generate a cline when graded selection favors different genotypes in different geographic regions. In these cases, the balance between selection and gene flow results in a cline, with gene flow acting as a homogenizing force among populations and opposing phenotypic divergence. Phenotypic plasticity can also create clines in the wild; however, phenotypic differences among populations for plastic traits disappear when individuals are reared in a common environment (for example, Maherali *et al.*, 2002). That is, clines generated directly by the environment do not necessarily involve genetic differentiation.

In animals, clinal variation is often observed for body color. For example, in humans, skin color is darkest at the

equator, with decreasing melanin in populations located toward the poles (Jablonski and Chaplin, 2000); in deer mice, coat color varies across Florida and Alabama, with the lightest phenotypes found closest to the Gulf of Mexico (Mullen and Hoekstra, 2008); and in the flat periwinkle snail, shell color varies in the Gulf of Maine, with the darkest shells found in the most northern, coolest waters (Phifer-Rixey *et al.*, 2008). Each of these clines appears to be adaptive, with selection pressures, including ultraviolet penetration, camouflage and thermoregulation, respectively. In *Drosophila*, pigmentation clines have been reported for *Drosophila melanogaster* (for example, David *et al.*, 1985; Pool and Aquadro, 2007; Parkash *et al.*, 2008), *D. simulans* (Capy *et al.*, 1988), the *D. dunnii* species subgroup (Hollocher *et al.*, 2000; Brisson *et al.*, 2005) and other *Drosophila* species (reviewed by Rajpurohit *et al.*, 2008). These clines correlate with both geographic (that is, latitude, altitude) and climatic (that is, temperature, humidity) factors. Laboratory studies in *D. melanogaster* and *D. polymorpha* show differences in desiccation resistance between color morphs (Kalmus, 1941; Brisson *et al.*, 2005; Rajpurohit *et al.*, 2008; Parkash *et al.*, 2009a,b), whereas studies in other insects show an effect of pigmentation on thermoregulation (for example, Watt, 1969; Brakefield and Willmer, 1985). *Drosophila* pigmentation is also known to be a plastic trait affected by environmental factors, such as food and temperature (for example, Gibert *et al.*, 2007).

This study examines the geographic distribution of body color in *D. americana*, a member of the *virilis* species group. The ancestor of *D. americana* colonized North

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America at least three million years ago and the species appears to have maintained a relatively stable, large effective population size since that time: patterns of codon usage in *D. americana* are more consistent with a theoretical population genetic 'equilibrium' than they are in the more commonly studied *D. melanogaster* (Maside *et al.*, 2004; Haddrill *et al.*, 2005). Consistent with this observation, previous studies of *D. americana* suggest extensive gene flow among populations (McAllister, 2002, 2003; Vieira *et al.*, 2003; McAllister and Evans, 2006; Schäfer *et al.*, 2006; Morales-Hojas *et al.*, 2008). Despite these signs of genetic homogeneity, however, 'a yellowish western group and a blackish eastern group' have been reported within this species (Throckmorton, 1982, p 239). These 'western forms' were collected primarily from Kansas, Nebraska, South Dakota and Montana (Hsu, 1951). *D. novamexicana*, the closest relative of *D. americana* (Caletka and McAllister, 2004; Morales-Hojas *et al.*, 2008), has even lighter and more yellow pigmentation than the western strains of *D. americana*, and has been collected from Arizona, Colorado, Utah and New Mexico (Throckmorton, 1982, p 239), suggesting a trans-species pigmentation cline that extends longitudinally across the United States.

Here, we provide the first quantitative description of the pigmentation cline in *D. americana* by measuring body color in 93 isofemale lines collected from 17 sites that span much of the latitudinal and longitudinal ranges of *D. americana*. Two different methods for quantifying pigmentation were used, one provides visual documentation and the other allows high-throughput scoring of live flies. Pigmentation differences among lines and among collection sites are shown to correlate with the longitude, which in turn correlates with the relative humidity. Patterns of sequence variation suggest extensive gene flow throughout the species range (consistent with previous studies) and reject an isolation-by-distance model of cline formation. We explore the hypothesis that differences in relative humidity among collection sites promote cline formation by testing for an effect of *D. americana* pigmentation alleles (that have been introgressed into *D. novamexicana*) on desiccation resistance. In contrast to studies of other *Drosophila* species (for example, Brisson *et al.*, 2005; Parkash *et al.*, 2009a b), we find no effect of pigmentation on desiccation resistance. We conclude by comparing these results with pigmentation clines observed in other *Drosophila* species.

Materials and methods

Fly strains

Two different strategies were used to measure pigmentation in *D. americana*. In 'dataset A', 13 isofemale lines, derived from 11 broadly distributed geographic locations in the central and eastern United States, were analyzed to provide a species-wide assessment of variability (Supplementary Table 1). Four of these lines, which were obtained from the *Drosophila* Species Stock Center (Tucson, AZ, USA), were established ~50 years ago from a single female fly captured at different collection sites. The remaining nine lines were established from female flies collected between 1999 and 2003 at seven other locations. With the exception of one site (Duncan, NE, USA), each of the collection sites included in dataset

A is represented by only a single isofemale line. These lines capture the breadth of pigment variation over the geographic range of the species. 'Dataset B' contains a deeper sampling of fewer sites (that is, 80 isofemale lines from eight different locations), with lines established from flies collected in June and September of 2007 (Supplementary Table 1). Collection sites in dataset B form a coarse longitudinal transect extending between 82° 98'W longitude and bounded by 38° 43'N latitude. Isofemale lines from localities near the eastern and western extremes of the transect (OR and DN, respectively) are included in both datasets; however, different isofemale lines from these collection sites are used in datasets A and B.

All fly stocks were maintained on standard yeast-glucose medium at 20–22 °C. Before pigmentation scoring, three male and three female flies were placed into a vial and their offspring raised at 20 °C. (Controlling the number of parents in each vial resulted in a similar larval density among genotypes.) Flies were collected within 3 days of eclosion and aged 1 week to allow body color to stabilize. All isofemale lines within dataset A or dataset B were reared simultaneously, under identical conditions (that is, light, humidity, temperature, batch of media) to minimize the effect of environmental differences among genotypes.

Quantifying pigmentation

For dataset A, dorsal abdominal pigmentation of each isofemale line was measured in five male and five female flies (aged 7–10 days) that had been placed in a 10:1 ethanol:glycerol mixture and stored at room temperature for 1 h to 1 month. The storage time of each individual was variable within each line and did not differ systematically among lines. We found that abdominal pigmentation is visually stable over this time window under these conditions. The dorsal abdominal cuticle was dissected from each fly, all underlying tissue was removed and the single layer of adult cuticle was mounted in Hoyer's solution. All mounted cuticles were imaged using a Scion 1394 (Frederick, MD, USA) camera under constant lighting conditions. Body color was quantified for each fly by using Image J (NIH, Bethesda, MD, USA) to calculate the average median pixel intensity of 20 randomly selected (and non-overlapping) regions in gray-scale images of the dorsal abdominal cuticle from segments A3, A4 and A5, using a measurement scale that ranged from 0 (black) to 255 (white) (Supplementary Figure 1). The mean and median coefficients of variation for individual flies were both 6%. A subset of samples was also analyzed using color images and found to provide similar discrimination among phenotypes to their gray-scale counterparts.

For dataset B, dorsal abdominal pigmentation of each isofemale line was measured in five male and five female flies, aged 7–10 days, using a custom-built R-series Fiber Optic Reflection Probe with a 50- μ m diameter fiber, an LS-1 Tungsten Halogen Light Source and a USB4000 Spectrometer (Ocean Optics Inc., Dunedin, FL, USA). The reflection probe contained six fiber optic wires that transmitted light to the fly cuticle and a seventh, central, fiber optic wire that transmitted light reflected off the sample to the spectrometer. The tip of the probe was encased by a custom-built shield constructed by the

instrument shop in the chemistry department at the University of Michigan, following the blueprint described at <http://www.lifesci.ucsb.edu/~endler/OceanOpticsList.pdf>. This probe shield ensures a constant distance (~1 cm) and angle (45°) between the fly cuticle and probe among measurements (Uy and Endler, 2004). The diameter of the probe tip (~0.7 mm) is approximately half of the anteroposterior length of one *D. americana* dorsal abdominal segment (that is, tergite).

After calibrating the SpectraSuite Spectroscopy Operating Software (Ocean Optics) with a WS-1 Diffuse Reflection Standard (Ocean Optics), the spectral reflectance of visible light (ranging from 0 to 100%) was recorded from five non-overlapping regions of dorsal abdominal cuticle (all located within segments A3, A4 and A5) from each fly. All measurements were collected over 2 consecutive days, with the isofemale lines scored in random order. Reference spectra taken from four dissected and mounted *D. americana* abdominal cuticles with varying pigmentation intensities were found to be similar on both days. Light from 610 to 660 nm wavelengths provided the greatest discrimination among the lightest and darkest control cuticles (Supplementary Figure 2), and custom Perl scripts were used to calculate the average reflectance of light in this range from each reflectance spectrum. In general, replicate measurements from the same fly were similar (mean and median coefficients of variation were 16 and 13%, respectively); however, extreme outliers were occasionally observed, which most likely resulted from the misalignment of the probe tip with the fly cuticle. To reduce the impact of these outliers, the median value from each fly (rather than the mean) was used for analysis.

DNA sequence variation and population genetic analysis
Genomic DNA was extracted from a single male fly from each isofemale line using the 'squish prep' protocol (Gloor *et al.*, 1993). For dataset A, regions from the following genes were amplified and sequenced in all lines except FP, which died before molecular analysis: *cytochrome b* (*cytB*, mitochondrial, 619 bp), *transformer* (*tra*, nuclear, 839 bp), *bazooka* (*baz*, nuclear, 575 bp), *l(1)G0007* (nuclear, 513 bp). In dataset B, the 839-bp region from the *tra* gene was successfully amplified and sequenced in 34 of the 80 isofemale lines, including at least three lines from each collection site. Sequences of primers used for both amplification and sequencing are available upon request. Sequences were assembled and aligned using CodonCode Aligner (Dedham, MA, USA) and manually validated by PJW for dataset A and by DCY for dataset B. They are available through Genbank with the following accession numbers: GU299293–GU299340 (dataset A) and GU248275–GU248308 (dataset B). Seven of the 34 *tra* sequences from dataset B (WS07.14, DN07.52 × 41, NN07.08, II07.10, OR07.10, SC07.18, MK07.24) were heterozygous at 1–7 sites and were resolved into two haplotypes (both of which were included in the sequence analysis), using the PHASEv2.1 algorithm (Stephens and Donnelly, 2003) implemented in DnaSP v5.10.00 (Librado and Rozas, 2009).

The following measures of genetic variability were calculated for each gene region using DnaSP v5.10.00 (Librado and Rozas, 2009): the number of segregating sites (*S*), haplotype diversity (*H_d*), nucleotide diversity

per site (π) and theta per site based on $S(\theta)$. For *tra* sequences of lines included in dataset B, we also calculated F_{st} and K_{st} (Hudson *et al.*, 1992) and assessed their significance using DnaSP and Arlequin v3.11 (Excoffier *et al.*, 2005), respectively. DnaSP was also used to calculate the test statistics Tajima's *D* and Fu and Li's *D**, and their statistical significance was determined using the distributions provided in the original descriptions of these statistics (Tajima, 1989; Fu and Li, 1993), as well as using 10 000 coalescent simulations based on summary statistics of the observed samples. Pair-wise genetic distances among all strains were calculated for each gene using the Tamura–Nei distance model of nucleotide substitutions (Tamura and Nei, 1993), as implemented in MEGA v4.0.2 (Kumar *et al.*, 2004). Sites with missing data or gaps were excluded from all analyses.

Statistical analyses

Pigmentation was analyzed primarily using PROC MIXED in SAS v9.1 (Cary, NC, USA), with all models described below fitted using restricted maximum likelihood. For dataset A, pigmentation measurements were fitted to the following model to test for effects of line and sex:

$$Y_{ijkl} = L_i + S_j + SL_{ij} + ISL_{ijk} + e_{ijkl}$$

where Y_{ijkl} is the mean pigmentation intensity for cuticle region *l*, from individual *k*, of sex *j*, from line *i*. *L* and *S* are fixed effects of isofemale line and sex, respectively; *I* is the random effect of individual within each sex × line combination; and e_{ijkl} is the residual error. Y_{ijkl} was weighted by the area of the cuticle region analyzed, with larger regions weighted more heavily than smaller regions. For each line (L_i) and each sex within each line (SL_{ij}), the least-squares mean and 95% confidence interval were calculated. Least-squares means were compared among lines using the Tukey's honestly significant difference *post hoc* test.

For dataset B, pigmentation measurements, consisting of a single (median) pigmentation score per fly, were fitted to the following model:

$$Y_{ijkl} = P_i + S_j + SP_{ij} + LSP_{ijk} + e_{ijkl}$$

where Y_{ijkl} is the pigmentation score for individual *l*, from isofemale line *k*, of sex *j*, from geographic population *i*. *P* and *S* are fixed effects of population and sex, respectively; *L* is the random effect of line within each population by sex combination; and e_{ijkl} is the error among pigmentation measures from individuals derived from the same isofemale line. For each population (P_i) and each sex within each population (SP_{ij}), the least-squares mean and 95% confidence interval were calculated. Least-squares means were compared among populations using Tukey's honestly significant difference *post hoc* test.

To test for geographic trends in pigmentation, we fitted both datasets to the following model:

$$Y_{jkl} = T_j + G_k + e_{jkl}$$

where Y_{jkl} is the least-squares mean pigmentation intensity for each line *l* collected from latitude *j* and longitude *k*. *T* and *G* represent the continuous covariates of latitude and longitude, respectively. For dataset A, only the intermediate of the three lines from Duncan, NE

was used, to avoid over-weighting data from this location. Male and female flies were analyzed separately for each dataset, because a significant effect of sex was detected (see Results section).

To test for evidence of isolation-by-distance model, we used a Mantel test to compare genetic and geographic distances among lines in dataset A and populations in dataset B. This test was conducted using the web-based Isolation-by-distance Web Service software v3.15 (Jensen *et al.*, 2005), available at <http://ibdws.sdsu.edu/>. Geographic distances for this test were measured in kilometers and calculated based on longitude and latitude of collection sites using the web-based software developed by Dr John Byers (US Arid-Land Agricultural Research Center, USDA-ARS, <http://www.chemical-ecology.net/java/lat-long.htm>). This analysis was also performed using geographic distances measured in degrees longitude. Genetic distances were calculated as described in the DNA sequence variation section above. Mantel tests were performed using both the raw genetic distances and the logarithm of genetic distances. Significance was assessed using 1000 permutations of the genetic and geographic distances, conducted by the Isolation-by-Distance Web Service software.

Desiccation resistance

Interspecific introgression lines were used to test specifically whether alleles that affect pigmentation have a corresponding effect on desiccation resistance. As described in Wittkopp *et al.* (2009), lines were constructed by crossing *D. americana* female flies to male flies of their lightly pigmented sister species, *D. novamexicana*, and backcrossing the resulting F₁ hybrid female flies to *D. novamexicana* male flies. Backcrossing was continued for 10 consecutive generations, with a single female heterozygous fly for the *D. americana* and *D. novamexicana* alleles of pigmentation genes *tan* and *ebony* genes mated to a *D. novamexicana* male fly in each generation. The introduction of either the *tan* or *ebony* genomic region from *D. americana* into *D. novamexicana* was sufficient to cause a visible darkening of pigmentation, with flies carrying *D. americana* alleles for both quantitative trait loci (QTLs) being visibly darker than those carrying *D. americana* alleles for either QTL region alone (Wittkopp *et al.*, 2009). Using these introgression lines, we constructed sex-specific pairs of genotypes with significant differences in pigmentation. The two male genotypes were both hemizygous for the *D. americana tan* QTL allele, but differed by the presence or absence of the *D. americana ebony* QTL allele, resulting in 'dark' and 'light' pigmentation phenotypes, respectively. Similarly, the two female genotypes were both heterozygous for the *D. americana tan* QTL allele, but differed by the presence or absence of the *D. americana ebony* QTL allele, again, resulting in 'dark' and 'light' pigmentation phenotypes, respectively.

Desiccation resistance was measured by placing 7- to 10-day-old virgin male and female flies into 5-ml Polystyrene round-bottom vials with mesh caps (BD Falcon, Bedford, MA, USA), which were stored in a 5.7-l plastic snap-lid container (Rubbermaid) with 200 g of Drierite (8 mesh), sealed with parafilm, and stored at 20 °C. A control container was prepared in the same manner, with the substitution of a moist paper towel for

the Drierite. Wired indoor/outdoor hygrometers (Radio-Shack) were used to monitor the relative humidity in each container: the desiccant container maintained a relative humidity level of <20% (the minimum detectable with the hygrometer) throughout the experiment, whereas the control container maintained an average of 85% relative humidity. Each container held 10 replicate vials of female flies and eight replicate vials of male flies, with each vial containing three 'light' and three 'dark' flies of the same sex. Beginning 15 h after placing the vials in the box, the number of dead flies (assessed by lack of visible movement when the vial was tapped) and the pigmentation of each dead fly (light or dark) were recorded every hour until all flies in the desiccation group died (50 h). Survival curves were compared using a non-parametric log-rank test, which compares the observed numbers of deaths at each time point between samples.

Results

To characterize the geographic distribution of body color in *D. americana*, we examined two distinct sets of isofemale lines. The first ('dataset A'), which contained a single isofemale line from each of 11 populations that span the known east-west range of *D. americana* (Throckmorton, 1982), was used to provide an overview of pigmentation differences across the species' range. The second ('dataset B'), which contained multiple isofemale lines derived from each of eight populations representing a coarse longitudinal transect through the central region of the species range, was used to assess body color variation within and between collection sites. The geographically extreme populations from dataset A were not included in dataset B because only a single isofemale line was available from these sites. Figure 1 and Supplementary Table 1 describe the collection sites and individual isofemale lines in more detail.

Quantitative metrics for adult body color in *Drosophila*

Drosophila pigmentation is typically analyzed using a subjective and arbitrary scoring scale based on visual assessments of pigmentation (for example, Hollocher *et al.*, 2000; David *et al.*, 2002; Wittkopp *et al.*, 2003b; Brisson *et al.*, 2005). Although these measurements are generally consistent for a single observer under controlled lighting conditions, discriminating among subtle gradations of body color is challenging for even the most experienced researcher. A preliminary visual assessment of pigmentation among isofemale lines of *D. americana* revealed obvious differences between the lightest and darkest lines, with subtle variation in intermediate body colors that we were unable to reliably and consistently classify by eye. Therefore, we concluded that an objective and quantitative method of pigmentation scoring was essential for describing the geographic distribution of body color in *D. americana*.

Two quantitative methods for scoring *Drosophila* pigmentation were developed and used in this study. The first method, which was applied to the 13 isofemale lines in dataset A, involved dissection of dorsal abdominal cuticles from preserved flies (five males and five females per line) followed by imaging and computational analysis of digital images from each individual cuticle. This method produced semipermanent samples

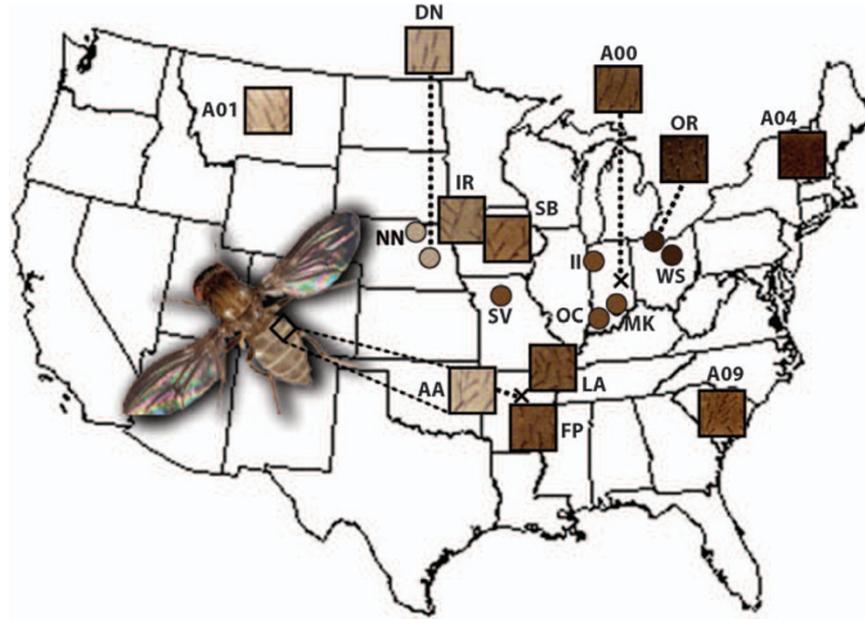


Figure 1 Body color in *D. americana* varies with longitude. (a) For all collection sites included in dataset A, a representative image of a region of dorsal abdominal cuticle is shown. All images are from male flies, and the DN2 line is shown for the Duncan, NE collection site. Collection sites included in dataset B are indicated with colored circles, wherein the circle approximates the cuticle color of flies from the collection site. As described in Supplementary Table 1, a non-overlapping set of isofemale lines from Duncan, NE (DN) and Ottawa National Wildlife Refuge, OH (OR) are included in datasets A and B.

and pictures of isolated body cuticles (Figure 1); however, the dissection, mounting and imaging was labor-intensive and time-consuming, making it impractical for analyzing multiple individuals from each of the 80 isofemale lines included in dataset B. To overcome this technical hurdle, we adapted a custom spectrometry system that allowed us to measure rapidly the pigmentation of live (but anesthetized) flies. Using this method, we quantified the pigmentation of 800 flies (five males and five females from each of 80 lines) for dataset B in only 2 days.

The distribution of body color variation within *D. americana*

To test for differences in pigmentation between sexes and among collection sites, measurements from datasets A and B were fitted separately to linear mixed models (see Materials and methods). Significant differences in pigmentation were observed among collection sites in both dataset A ($F = 12.46$, $P < 0.0001$) and dataset B ($F = 36.01$, $P < 0.0001$). *Post hoc* analysis of these data identified four statistically distinct pigmentation groups in dataset A and two statistically distinct groups in dataset B (Table 1); all locations within one group showed statistically significant differences in pigmentation from locations in all other groups. *D. americana* female flies were found to be slightly, but significantly, lighter in color than *D. americana* male flies in both datasets (dataset A: $F = 4.32$, $P = 0.0405$; dataset B: $F = 10.83$, $P < 0.0001$), although this sexual dimorphism is not visually apparent under a dissecting microscope and has not been recognized previously. Male flies were 5.5% darker than female flies in dataset A and 12.5% darker than female flies in dataset B, which may be partially due to colored tissues underlying the cuticle in dataset B that were

removed for dataset A. To examine the geographic distribution of different pigmentation phenotypes, we fitted the pigmentation measures to a linear model that included the latitude and longitude of the collection site as covariates. A highly significant effect of longitude was observed for both datasets, whereas the effect of latitude showed no significant effect in either case (Table 2). Manual inspection of the geographic distribution of pigmentation phenotypes suggests that the longitudinal gradient may actually be nonlinear, with the largest change in pigmentation occurring near 90° west longitude; however, nonlinear models fit to our data with SAS v9.1 (proc NLIN) and Cfit (Gay *et al.*, 2008) failed to converge.

Clinal variation is inconsistent with a neutral isolation-by-distance model

The observed longitudinal gradient of pigmentation in *D. americana* may be caused by local adaptation or genetic drift with geographically limited migration (isolation-by-distance). These two different evolutionary processes can be distinguished by comparing the spatial distribution of pigmentation with the spatial distribution of genetic variation. Specifically, clines resulting from isolation-by-distance are expected to show a positive correlation between genetic and geographic distances at neutral loci, whereas clines resulting from natural selection despite ongoing gene flow are not. To distinguish between these hypotheses, we surveyed sequence variation among isofemale lines in both datasets.

For dataset A, regions from the *cytB*, *baz*, *l(1)G0007* and *tra* genes were sequenced in 12 of the 13 isofemale lines; no sequences were obtained from the FP isofemale line because the stock died before molecular genetic analysis. According to Flybase (Drysedale and Consortium, 2008),

none of the loci surveyed affects pigmentation. Neutral-ity tests based on Tajima's *D* and Fu and Li's *D**, both of which compare the observed distribution of polymorph-ism with a distribution expected under a neutral model, were consistent with neutrality (Table 3), suggesting that variation of the sequenced loci should reflect gene flow among the populations sampled. A region of sequence from the *tra* gene obtained from 34 isofemale lines from

dataset B, including at least three lines from each collection site, was also consistent with neutrality (Table 3). Furthermore, pair-wise F_{st} and K_{st} for sequences from dataset B showed no significant differ-ences between populations after correcting for multiple tests (Table 4), and there was also no evidence of population subdivision when all populations in dataset B were considered together ($K_{st} = 0.018, P = 0.20$). Finally, F_{st} and K_{st} were also not significant for either dataset when sequences were compared between 'light' and 'dark' pigmentation classes (Table 5).

Table 1 Pigmentation intensity among isofemale lines and collec-tion sites

Source	Longitude	LSM	Group
<i>Dataset A</i>			
A01	105.2	163.41	a
AA	91.4	161.39	a
DN00.2	97.5	149.36	a
DN00.4	97.5	140.96	a, b
DN01.12	97.5	128.64	b
SB	91.2	130.76	b
FP	91.1	127.97	b, c
IR	91.7	126.18	b, c, d
A09	78.9	113.44	c, d
A00	85.6	110.75	c, d
LA	90.8	109.47	c, d
A04	73.4	106.29	d
OR	83.2	98	d
<i>Dataset B</i>			
NN	98.1	-14.53	a
DN	97.5	-15.03	a
OC	87.3	-17.76	b
MK	85.8	-18.00	b
SV	93.2	-18.57	b
II	87.5	-18.62	b
WS	82.0	-18.85	b
OR	83.2	-19.51	b

Abbreviation: LSM, least-squares mean.

For each isofemale line ('Source') analyzed in dataset A, the longitude of the collection site (in degrees W), the least-squares mean (LSM) from the mixed model described in Materials and methods and the pigmentation group (based on Tukey's *post hoc* test) are shown. A full description of each 'Source' is provided in Supplementary Table 1. The same metrics are shown for dataset B, except that LSMs are reported for each collection site rather than for each isofemale line and are based on a slightly different mixed model, as described in Materials and methods. Note that different pigmentation-scoring techniques were used for datasets A and B; thus, the LSM values reported for the two datasets are on different scales. Tukey's *post hoc* analysis was performed separately for the two datasets; thus, there is no implied relationship between groups with the same label (for example, 'a') in datasets A and B.

Sequences from both dataset A and dataset B were used separately to test a model of isolation-by-distance by using a Mantel test to compare pair-wise genetic and geographic distances. In dataset A, we found no significant relationship between genetic and geographic distances, regardless of whether geographic distance was measured in kilometers (Table 6) or degrees longi-tude (data not shown). Similarly, sequences from dataset B were also inconsistent with an isolation-by-distance model, regardless of whether the pair-wise Tamura-Nei genetic distance among isofemale lines or F_{st} between populations was used to estimate genetic distance or whether kilometers (Table 6) or degrees longitude (data not shown) was used to measure geographic distance.

Lacking clear evidence for genetic differentiation among collection sites, which is consistent with previous studies that also failed to find evidence of population structure in *D. americana* using different samples (McAll-ister, 2003; Vieira *et al.*, 2003; Maside *et al.*, 2004; McAllister and Evans, 2006; Schäfer *et al.*, 2006; Mor-ales-Hojas *et al.*, 2008), as well as the rejection of an isolation-by-distance model by both datasets, we con-clude that the observed clinal variation for pigmentation in *D. americana* is unlikely to be the product of genetic drift in distinct populations, but rather is more likely maintained across the species range by natural selection for locally adaptive phenotypes.

Differential selection for desiccation resistance unlikely to explain the pigmentation cline

As described above, we found that pigmentation in *D. americana* correlates much more strongly with longitude than with latitude (Figures 2a and b and Supplementary Figure 3). Further analysis showed that pigmentation in dataset B also correlates significantly with altitude (Figure 2c), although this is not surprising given that

Table 2 Pigmentation varies significantly by longitude, but not latitude

	Dataset A		Dataset B	
	Female flies	Male flies	Female flies	Male flies
Effect of latitude	F = 0.27 P = 0.61	F = 0.57 P = 0.47	F = 0.50 P = 0.48	F = 0.10 P = 0.75
Effect of longitude	F = 14.66 P = 0.005	F = 6.50 P = 0.034	F = 22.39 P < 0.0001 ^a	F = 15.66 P = 0.0002
Mean pigment intensity	131.8 ± 2.35	124.5 ± 2.63	-16.44 ± 0.189	-18.47 ± 0.192

F- and P-values were obtained for the following model: $Y_{jkl} = T_j + G_k + e_{jkl}$ (described more fully in Materials and methods). Mean pigmentation scores for male and female flies are given in the bottom row, ± standard error.

^aPigmentation was measured using different scales for dataset A and dataset B; however, larger ordinal values correspond to lighter coloration in both cases.

Table 3 Genetic variation among isofemale lines

Gene	Length (bp)	S ^a	Hd ^b	Π ^c	Θ ^d	Genomic location	Tajima's D ^e	Fu and Li's D ^f
Dataset A								
<i>baz</i>	575	23	0.85	0.014	0.014	X-linked	0.25	-0.13
<i>cyt b</i>	619	17	1.00	0.007	0.009	Mitochondrial	-1.05	-1.21
<i>l(1)G0007</i>	513	37	0.91	0.024	0.026	X-linked	-0.35	-0.39
<i>tra</i>	839	38	0.99	0.016	0.016	Autosomal	-0.26	0.05
Dataset B								
<i>tra</i>	549	51	0.99	0.015	0.021	Autosomal	-1.01	1.16

^aNumber of segregating sites.^bHaplotype diversity.^cNucleotide diversity per site.^dPer site based on S.^eP-values calculated using the beta distribution of D described in Tajima (1989) as well as with 10 000 coalescent simulations in DnaSP. All $P > 0.05$.^fP-value calculated using simulations in Fu and Li (1993) as well as with 10 000 coalescent simulations in DnaSP. All $P > 0.05$.**Table 4** Comparison of genetic diversity within and among populations

	DN	MK	NN	OC	OR	SC	WS	II
DN		-0.05	-0.05	-0.12	0.01	-0.03	0.00	-0.04
MK	-0.01		-0.01	-0.12	0.23	-0.03	0.09	-0.03
NN	-0.01	0.02		-0.08	0.02	-0.02	0.13	-0.04
OC	-0.05	-0.03	-0.03		-0.03	-0.04	0.02	-0.13
OR	0.05	0.08	0.05	0.03		0.05	0.10	-0.02
SC	0.01	0.01	0.02	0.01	0.08*		0.11	-0.11
WS	-0.03	0.06	0.03	-0.03	0.09*	0.03		0.04
II	-0.04	0.02	0.01	-0.04	0.02	-0.04	-0.05	

F_{st} (above diagonal) and K_{st} (below diagonal) are shown for *tra* sequences from dataset B. Two-letter abbreviations refer to collection sites, as defined in Supplementary Table 1. P -values for all F_{st} and K_{st} values are > 0.05 , except $K_{st(OR-SC)}$ and $K_{st(OR-WS)}$ (labeled with asterisks (*) in the table), which have P -values of 0.04 and 0.02, respectively; both of these values are nonsignificant after a sequential Bonferroni correction for multiple ($n = 56$) tests.

Table 5 Comparison of genetic diversity between pigmentation classes

	F_{st}	K_{st}
Dataset A		
<i>baz</i>	0.07 (-0.02)	0.03 (0.0005)
<i>cytB</i>	-0.08 (-0.09)	-0.05 (-0.04)
<i>l(1)G0007</i>	-0.04 (0.07)	0.005 (0.03)
<i>tra</i>	0.003 (-0.006)	-0.01 (-0.003)
Dataset B		
<i>tra</i>	0.0008	-0.002

For each dataset, sequences were divided into 'light' and 'dark' classes using the Tukey groups shown in Table 1. For dataset A, the light class included isofemale lines from group a (that is, A01, AA, DN2), whereas the dark class included isofemale lines from groups b, c, and d (that is, DN12, SB, IR, A00, A04, A09, LA and OR). DN4, which was assigned to groups a and b by the Tukey analysis, was treated in separate analyses as a member of the light and dark classes, with the results from including DN4 in the dark class shown in parentheses. For dataset B, the light class included all sequences from the NN and DN populations, whereas the dark class included all sequences from the OC, MK, SV, II, WS and OR populations. In all cases, F_{st} and K_{st} values were consistent with a null hypothesis of no genetic differentiation between pigmentation classes ($0.15 > P$ -value > 0.95).

longitude and altitude are themselves correlated for the collection sites examined ($R^2 = 0.52$). In other *Drosophila* species, latitude and altitude are the primary correlates with pigmentation clines (see Discussion). Differences in temperature and relative humidity among collection sites, which presumably affect thermal and desiccation tolerances, respectively, are the most commonly invoked selective agents for the formation and maintenance of pigmentation clines in *Drosophila* (reviewed by True, 2003, Wittkopp *et al.*, 2003a and Rajpurohit *et al.*, 2008), and among all collection sites examined in this study, relative humidity correlates more strongly with longitude ($R^2 = 0.37$) than with latitude ($R^2 = 0.08$), whereas the opposite is true of temperature—it correlates more strongly with latitude ($R^2 = 0.96$) than longitude ($R^2 = 0.37$). Despite these correlations, no significant direct correlation was found between pigmentation and temperature or relative humidity in either dataset (Figures 2d and e).

Associations between pigmentation and humidity have been reported in at least seven *Drosophila* species (Brisson *et al.*, 2005; Rajpurohit *et al.*, 2008; Parkash *et al.*, 2008, 2009b). In all cases, darker flies were collected from less humid environments. Interestingly, *D. americana* appears to show the opposite pattern: lighter flies were collected from less humid environments (Figure 2e), suggesting that distinct selective mechanisms may be operating in *D. americana*. To test the effect of pigmentation on desiccation resistance as specifically as possible, we compared desiccation resistance between sex-specific pairs of introgression lines that differed dramatically for pigmentation, but minimally for genotype. This experimental strategy minimizes the possibility that correlated variation with no effect on pigmentation causes differences in desiccation resistance through other physiological mechanisms. As described in the Materials and methods, the introgression lines used for this analysis contained genetic material from both *D. americana* and its closest relative, *D. novamexicana* (Wittkopp *et al.*, 2009), with the dark and light genotypes examined differing only by the presence or absence, respectively, of the *D. americana* allele of *ebony* and surrounding genes.

We measured desiccation resistance in each of these pigmentation classes using the same desiccation

Table 6 Evaluating a model of isolation-by-distance

	Genetic distance				Log(genetic distance)			
	Z	r	P ^a	R ^b (RMA)	Z	r	P ^a	R ^b
Dataset A								
<i>Bazooka</i>	983	-0.11	0.76	0.0128	-154804	-0.23	0.97	0.0506
<i>Cytochrome b</i>	503	0.17	0.19	0.0296	-151664	0.13	0.24	0.0163
<i>Transformer</i>	1055	-0.18	0.80	0.0341	-131479	-0.22	0.92	0.0475
<i>I(1)G0007</i>	1725	-0.02	0.51	0.0003	-127491	-0.09	0.73	0.0087
Dataset B								
<i>Transformer</i>	7640	0.01	0.39	9.34×10^{-5}	-949507	0.04	0.17	1×10^{-4}
<i>Transformer^b</i>	-1567	0.005	0.43	2.59×10^{-5}	-9×10^{21}	-0.02	>0.99	5×10^{-4}

Mantel tests for correlation between genetic and geographic distance (measured in kilometers).

^aP-values are one sided and based on 1000 permutations. The null hypothesis is that the correlation coefficient is ≤ 0 .

^bGenetic distance measured by pairwise F_{st} rather than the Tamura-Nei genetic distance.

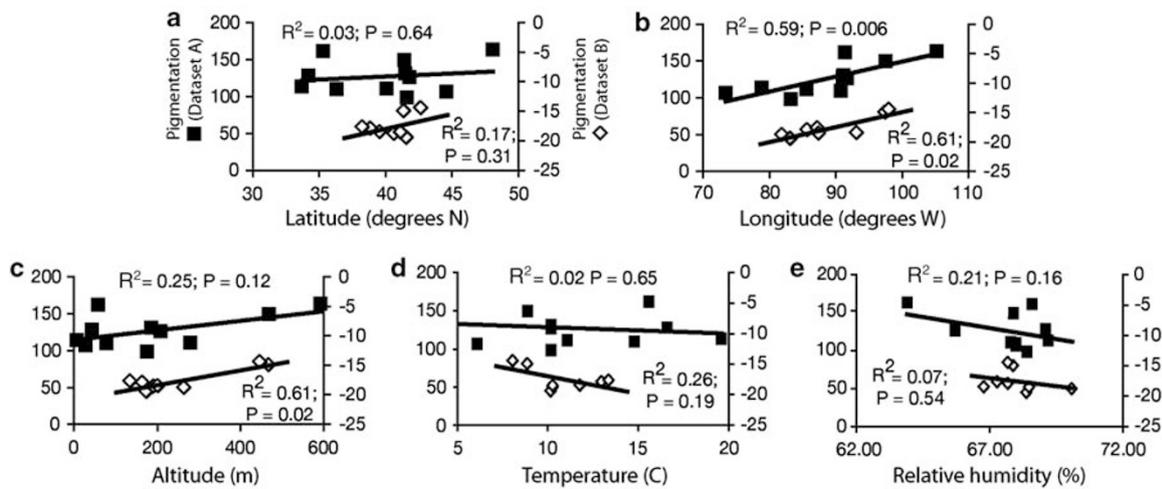


Figure 2 Geographic and environmental correlates with pigmentation. Line-specific least-squares means for pigmentation from dataset A (closed boxes) and population-specific least-squares means for pigmentation from dataset B (open diamonds) are plotted against the latitude (a), longitude (b), altitude (c), temperature (d) and relative humidity (e) for each collection site. The R^2 values are shown for each comparison along with their associated P -values (determined using proc reg in SAS), with values for dataset A in the top part of each panel and values for dataset B in the lower part of each panel. Note that dataset A encompasses a larger range for each of the variables analyzed than dataset B.

tolerance assay that was used to show differences in desiccation resistance between pigmentation classes of other *Drosophila* species (Brisson et al., 2005; Rajpurohit et al., 2008; Parkash et al., 2008, 2009a, b). Surprisingly, we found no significant difference in desiccation resistance between light and dark flies of either sex (Figure 3). That is, flies of the same sex had similar survival times (as measured by a log rank test) under desiccating conditions, regardless of whether they had light or dark pigmentation (males: $\chi^2 = 0.3$, degrees of freedom = 1, $P = 0.58$; females: $\chi^2 = 1.4$, degrees of freedom = 1, $P = 0.2$). Significant differences in survival time were observed between the sexes, however. Male flies survived longer for both light ($\chi^2 = 21.2$, degrees of freedom = 1, $P = 4 \times 10^{-6}$) and dark ($\chi^2 = 27.4$, degrees of freedom = 1, $P = 2 \times 10^{-7}$) phenotypes, which may be caused by sexual dimorphism and/or differences in their X-chromosome genotypes. Sexual dimorphism for desiccation resistance has been reported in other *Drosophila* species as well, although female flies typically survive longer than male flies under desiccating conditions (Brisson et al., 2005; Matzkin et al., 2007).

Discussion

Pigmentation is one of the most variable traits in the genus *Drosophila*: differences in body color are common among individuals within a population, among populations of the same species and among closely related species. This study uses two objective methods of scoring pigmentation, one of which allows for high-throughput analysis, to provide a quantitative description of body color variation among geographic isolates of *D. americana*. A longitudinal gradient for pigmentation is described, with the lightest body color found in the western extent of the species range. The findings are consistent with previous references to variable pigmentation between western and eastern flies, which were previously recognized based solely on anecdotal observations. Moreover, this study revealed the existence of a slight sexual dimorphism characterized by more lightly pigmented female flies.

Patterns of *D. americana* sequence variation (observed in this and previous studies) indicate extensive gene flow among populations and are inconsistent with the

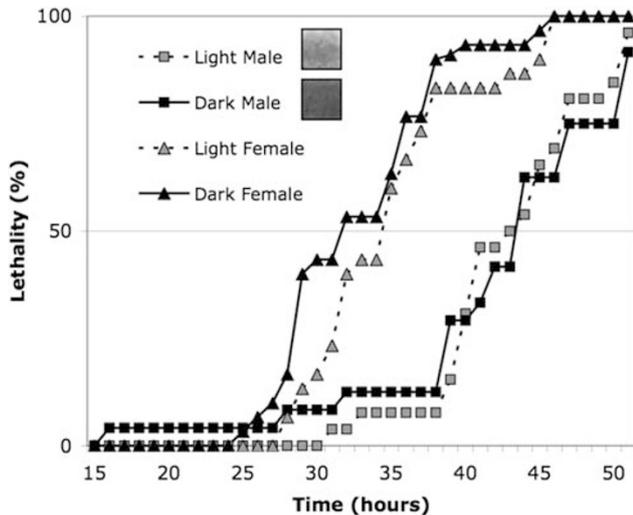


Figure 3 Body color has no effect on desiccation resistance. A time course of lethality under desiccating conditions is shown for both 'light' (gray, dotted lines) and 'dark' (black, solid lines) flies. Triangles represent female flies and squares represent male flies. (For a full description of the genotype of these flies, see Materials and methods.) Pigmentation of the dorsal abdominal cuticle from a 'light' and a 'dark' male fly is shown. Female flies in both pigmentation classes were slightly lighter than their male counterparts because the introgressed X-linked *D. americana* pigmentation allele(s) were hemizygous in male flies and heterozygous in female flies.

differentially pigmented forms being established by a neutral isolation-by-distance model of evolution. In contrast, Hsu (1952) identified several chromosomal inversions that differ in frequency between western and eastern populations. An inversion located distally on chromosome 2 contains the *ebony* locus that contributes to pigmentation differences (Wittkopp *et al.*, 2009). Thus, the recognition of geographically distinct populations on the basis of chromosomes and pigmentation is not entirely independent. The distinction between western and eastern populations is, however, not reflected in patterns of sequence variation throughout the genome. The presence of a pigmentation cline in *D. americana*, despite the homogenizing effects of gene flow, suggests that pigmentation differences observed among collection sites are adaptive and the product of natural selection. (However, it is also possible that genes affecting pigmentation are linked to genes affecting another trait that is locally adaptive, and pigmentation correlates with longitude as a result of linkage and clinal selection pressures for this other trait.) Differences in relative humidity exist across the species range that might favor different pigmentation phenotypes in different locations; however, laboratory assays failed to show any significant difference in desiccation resistance between flies with light and dark pigmentation. Below, we compare these results with pigmentation clines in other *Drosophila* species.

The longitudinal pigmentation cline of *D. americana* is atypical for *Drosophila*

In *D. melanogaster* populations from multiple continents, thoracic pigmentation correlates with latitude: flies from

higher latitudes have darker pigmentation (Munjal *et al.*, 1997; Parkash and Munjal, 1999; Parkash *et al.*, 2008). Darker thoracic pigmentation is also characteristic of high-altitude populations from India, which persist in an environment with lower relative humidity than low altitude populations (Parkash and Munjal, 1999; Parkash *et al.*, 2008). A similar relationship between thoracic pigmentation and relative humidity was observed for seasonal pigmentation variation of *D. melanogaster* in montane regions of India (Parkash *et al.*, 2009a). In sub-Saharan Africa, abdominal pigmentation of *D. melanogaster* correlates with latitude, but correlates even more strongly with altitude (Pool and Aquadro, 2007). *D. simulans*, a close relative of *D. melanogaster*, has much less variation for body color, yet still shows a weak correlation with latitude for thoracic pigmentation (Capy *et al.*, 1988).

In the *dunni* species subgroup, a latitudinal cline exists for abdominal pigmentation that includes multiple species and extends from Puerto Rico through the Lesser Antilles islands in the Caribbean (Heed and Krishnamurthy, 1959; Hollocher *et al.*, 2000). In contrast to studies of *D. melanogaster*, in which the darkest phenotypes are found at the highest latitudes, the darkest phenotypes in the *dunni* species group are found closest to the equator. Genetic analysis indicates that pigmentation differences among species in the *dunni* group are more likely to have been established by natural selection than through patterns of common ancestry among species (Hollocher *et al.*, 2000).

Considering that all of the previously described pigmentation clines in *Drosophila* correlate with latitude, the absence of a latitudinal cline and the discovery of a longitudinal pigmentation cline in *D. americana* are surprising. In *D. melanogaster*, the correlation between pigmentation and latitude appears to be explained largely by differences in altitude; however, we found that this is unlikely to be the case for *D. americana*. Among the North American sites sampled for this work, altitude shows a similar correlation with both latitude ($R^2 = 0.49$) and longitude ($R^2 = 0.52$), and in dataset A, which contains more comprehensive sampling of variation within *D. americana* than dataset B, pigmentation does not correlate significantly with altitude (Figure 2c). (Note that a significant correlation with altitude was observed for dataset B, however.)

D. americana shows unique relationships among pigmentation, relative humidity and desiccation resistance. Relative humidity (or aridity) is one of the most frequently invoked environmental correlates with pigmentation in *Drosophila*, and differences in desiccation resistance between light and dark pigmentation morphs have been reported for multiple species (reviewed by Rajpurohit *et al.*, 2008, True, 2003 and Wittkopp and Beldade, 2009). For example, in *D. melanogaster*, a laboratory assay showed that darker flies collected from natural populations survived longer under desiccating conditions (for example, Parkash *et al.*, 2008), with a similar pattern observed among seasonal morphs (Parkash *et al.*, 2009a). *D. polymorpha*, a close relative of the *dunni* species group that does not show an obvious pigmentation cline, is enriched for darker phenotypes in

warm, arid open areas in comparison with cooler, more humid covered forests (Brisson *et al.*, 2005). These darker forms of *D. polymorpha* were found to survive longer than their lighter counterparts under desiccating conditions in the laboratory. Indeed, darker body pigmentation has been shown to increase desiccation resistance in *D. nepalensis*, *D. takahashii*, *D. ananassae*, *D. jambulina* and *D. immigrans* (Rajpurohit *et al.*, 2008; Parkash *et al.*, 2008, 2009b). This increase in desiccation resistance appears to be caused by a slower rate of water loss in individuals with greater melanization (Brisson *et al.*, 2005; Rajpurohit *et al.*, 2008).

In light of these data, the presence of darker *D. americana* in more humid areas is surprising and suggests that the primary selective force promoting the pigmentation cline in *D. americana* might be different from that in other species. It is also possible that pigmentation has a different effect on desiccation resistance in different species. Consistent with this latter possibility, we observed no significant difference in desiccation resistance between light and dark forms of *D. americana*/*D. novamexicana* introgression lines. Our experiment used virtually the same assay for desiccation resistance as previous studies (that is, survival time in a desiccating environment); however, its design differed from previous work in two important ways. First, we compared defined genotypes derived from introgression lines rather than natural isolates or individuals from a segregating F₂ (or other recombinant) population. This allowed us to analyze flies that were genetically homogeneous within a pigmentation class and differed for only a single region of the genome between pigmentation classes, which greatly reduces the possibility that genetic variation affecting traits other than pigmentation contributes to differences in desiccation resistance. Second, we tested the effects of *D. americana* pigmentation alleles on desiccation resistance in the genetic background of its sister species, *D. novamexicana*. The *D. novamexicana* background and/or interactions between the two different species alleles might have altered the relationship between pigmentation and desiccation resistance; however, we see no reason to suspect that this is the case. The light pigmentation of *D. novamexicana* appears to be an extension of the *D. americana* longitudinal gradient (Throckmorton, 1982) and the two species retain many shared ancestral polymorphisms (Hilton and Hey, 1996; Morales-Hojas *et al.*, 2008; Wittkopp *et al.*, 2009).

In summary, we conclude that our data do not support the hypothesis that differences in relative humidity among collection sites cause selection for differences in desiccation resistance that are mediated by differences in body color. That said, our data are also insufficient to disprove such a hypothesis. The standard laboratory assay for desiccation tolerance is extremely crude: variation in relative humidity among wild populations is much less extreme than the difference between high and low humidity chambers set up in the laboratory and the phenotypes affected by humidity levels in the wild are likely much more subtle than death. Parkash *et al.* (2009a) have recently shown that desiccation stress alters mate choice, copulation duration and fecundity of *D. jambulina*, and we suspect that desiccation effects such as these have a much larger impact on fitness in the wild.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)