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# Vanishing Chromosomal Inversion Clines in *Drosophila subobscura* from Chile: Is Behavioral Thermoregulation to Blame?

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**ABSTRACT:** Chromosomal inversion clines paralleling the long-standing ones in native Palearctic populations of *Drosophila subobscura* evolved swiftly after this species invaded the Americas in the late 1970s and early 1980s. However, the new clines did not consistently continue to converge on the Old World baseline. Our recent survey of Chilean populations of *D. subobscura* shows that inversion clines have faded or even changed sign with latitude. Here, we investigate the hypothesis that this fading of inversion clines might be due to the Bogert effect, namely, that flies' thermoregulatory behavior has eventually compensated for environmental variation in temperature, thus buffering selection on thermal-related traits. We show that latitudinal divergence in thermal preference ( $T_p$ ) has evolved in Chile for females, with higher-latitude flies having a lower mean  $T_p$ . Plastic responses in  $T_p$  also lessen latitudinal thermal variation because flies developed at colder temperatures prefer warmer microclimates. Our results are consistent with the idea that active behavioral thermoregulation might buffer environmental variation and reduce the potential effect of thermal selection on other traits as chromosomal arrangements.

**Keywords:** Bogert effect, chromosomal inversion polymorphism, clinal variation, phenotypic plasticity, temperature, thermal preferences.

## Introduction

Swift latitudinal clinal variation in chromosomal inversion polymorphism has evolved in *Drosophila subobscura* since this species invaded South and North America from Europe in the late 1970s and early 1980s (Prevosti et al. 1985, 1988; Balanyà et al. 2003). These clines were generally parallel to the long-standing clines in the original popu-

lations (Krimbas and Loukas 1980; Menozzi and Krimbas 1992), thus providing compelling evidence for the adaptive significance of the inversion polymorphism in this species. Several lines of evidence suggest that temperature may be the underlying factor (reviewed in Rezende et al. 2010). First, cyclical seasonal changes consistent with the latitudinal patterns have been recorded in a long time-series experiment at one locality in northwestern Spain (Fontdevila et al. 1983; Rodríguez-Trelles et al. 1996). Second, cold-climate gene arrangements are retreating in frequency worldwide, presumably because of climate warming (Balanyà et al. 2006).

However, the actual role played by temperature is not without caveats (e.g., Santos et al. 2005), and other climatic variables may also be important (Rodríguez-Trelles and Rodríguez 1998). Periodic fluctuations in population mixing among geographically differentiated populations might induce cyclical changes, and range shifts of more equatorial populations toward higher latitudes might also partially explain the exceptionally fast worldwide shifts of gene arrangement frequencies in response to increasing temperature (Santos 2007; Balanyà et al. 2009; Rezende et al. 2010). An additional important point emphasized by Huey and Pascual (2009) is that latitudinal (seasonal) variation in body temperature ( $T_b$ ) of *D. subobscura* has always been assumed but that thermoregulatory behaviors (e.g., by modifying daily activity patterns and selecting favorable microclimates; Stevenson 1985) may mitigate or eliminate fluctuations in its thermal environment, so that little selection for temperature-related changes would occur. This is known as the "Bogert effect" (Huey et al. 2003) in honor of Bogert (1949), who first described how behavioral thermoregulation might compensate for environmental vari-

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ation in temperature, thus buffering selection on thermal physiology (see also Bartholomew 1958).

*Drosophila* flies can actively thermoregulate by choosing a preferred body temperature ( $T_p$ ) in a laboratory thermal gradient that presumably corresponds to their thermal optimum (Dillon et al. 2009). Recent work with *D. subobscura* has shown that flies carrying cold-climate gene arrangements tended to choose lower temperatures than their counterparts carrying warm-climate ones (Rego et al. 2010). However, flies can also modify their  $T_p$  in response to environmental cues, and plastic responses could be more important than actual genetic differences. The extent to which body temperature relates to ambient temperature ( $T_a$ ) can be visualized under three possible scenarios, or types of fly (see Huey et al. 2003): (1) a thermoconformer, or null fly, where  $T_b$  equals average environmental temperature; (2) a fly in which behavioral thermoregulation is fully compensatory, so that  $T_b$  remains approximately constant; and (3) a fly that seeks out its thermal optimum but whose capacity to thermoregulate behaviorally is only partial. The intensity of thermal selection will be the greatest in the first scenario and fully absent in the second scenario. In the likely more realistic final scenario, some selection will occur for traits that are directly related to temperature.

The actual association between  $T_p$  and latitude is difficult to predict. On the one hand, we could expect that the thermal optimum would eventually shift toward the environmental temperature at each locality to maximize performance (i.e., a negative relationship between  $T_p$  and latitude). On the other hand, countergradient selection (Gill et al. 1983), where individuals from colder environments tend to select higher temperatures in a laboratory thermal gradient, can also be expected, as plasticity in  $T_p$  may partially compensate for the thermodynamic constraints in metabolic and growth rates imposed by low temperatures (Angilletta et al. 2010). In fact, some studies have found negative (turtles: Ellner and Karasov 1993), positive (amphibians: Freidenburg and Skelly 2004; fishes: Fague et al. 2009), or no relationship at all (fishes: Gamperl et al. 2002; terrestrial isopods: Castañeda et al. 2004) between  $T_p$  and latitude. For *Drosophila virilis* and *Drosophila immigrans*, little or no geographic differences in  $T_p$  were found by Yamamoto (1994). However, the experimental flies had been maintained in the laboratory for several years, and rapid adaptation to laboratory conditions might compromise accurate microevolutionary inferences from comparative data collected from different populations (Matos et al. 2000, 2004). In their recent and comprehensive revision of thermal preference in *Drosophila*, Dillon et al. (2009, pp. 116–117) concluded, “There is also currently no general consensus on the plasticity of thermal preference in response to developmental and acclimation temperature.... Despite some evidence that thermal preference is heritable, few studies have demon-

strated evolved divergence in thermal preference in the lab or in the field.”

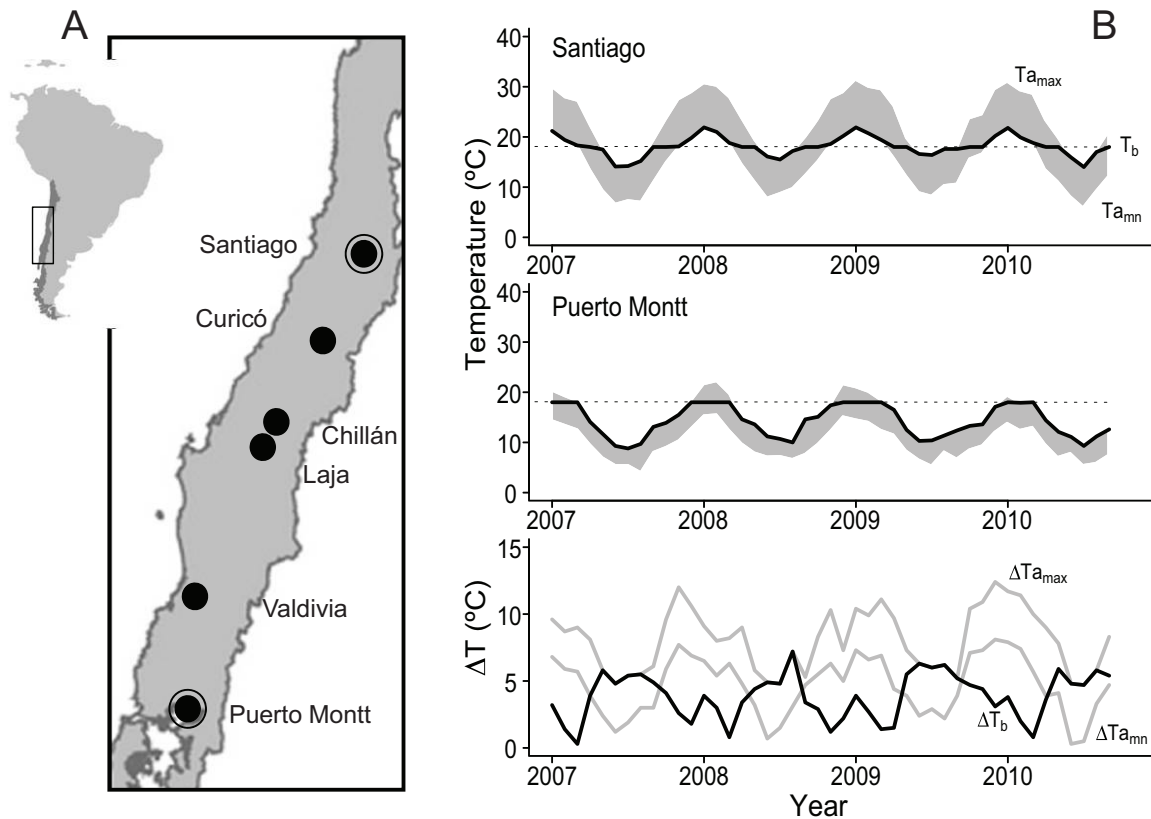
Our aim here is to test the suggestion put forward by Rego et al. (2010) that thermoregulatory behavior in *D. subobscura* decouples mean  $T_b$  and mean environmental temperature to somewhat mitigate the effect of variation in thermal environments along a latitudinal gradient. The main reason for our undertaking was that a recent survey in Chile shows that chromosomal inversion clines have faded or have even changed sign with latitude in a way that conflicts with the Old World baseline. Chilean populations of *D. subobscura* offer a particularly powerful system to analyze evolved divergence in  $T_p$  (a proxy for the thermal optimum; Dillon et al. 2009), because the history of invasion is well known (Ayala et al. 1989; Huey et al. 2005; Rezende et al. 2010). Briefly, fewer than 15 individuals (Pascual et al. 2007) from southwestern Europe (the most likely candidate region to be the source of the colonizing flies) crossed to Puerto Montt (Chile) in 1978, and in less than a year the species colonized much of the habitable coast of Chile. Despite this genetic bottleneck, laboratory natural-selection experiments using a stock of flies from Puerto Montt showed that the thermal optimum of *D. subobscura* diverged when flies were maintained in different constant thermal regimes (Santos 2007), which suggests that  $T_p$  can also evolve in nature in response to climate. Here, we show that evolved latitudinal divergence in  $T_p$  has indeed occurred in Chile for females, with higher-latitude flies having a lower mean  $T_p$ . However, plastic responses in  $T_p$  may buffer latitudinal variation in temperature, because flies developed at colder temperatures prefer warmer microclimates. These findings provide circumstantial evidence that plastic behavioral thermoregulation might mitigate thermal selection effects and inhibit further evolutionary change in chromosomal inversion polymorphism.

## Material and Methods

### *Fly Collections and Maintenance*

During the austral spring of 2010, adult flies of *Drosophila subobscura* were collected from six low-altitude sites along a latitudinal gradient in Chile (fig. 1A): Santiago (33°27'S, 70°42'W), Curicó (34°58'S, 71°13'W), Chillán (36°30'S, 72°06'W), Laja (37°12'S, 72°24'W), Valdivia (39°48'S, 73°14'W), and Puerto Montt (41°28'S, 72°57'W). Data of inversion frequencies from these populations had been periodically obtained ever since *D. subobscura* invaded Chile in the late 1970s (Prevosti et al. 1985, 1988; Balanyà et al. 2003).

Females from each population were individually placed in glass vials with David's killed-yeast *Drosophila* medium (David 1962) to establish isofemale lines. An  $F_1$  male from each progeny was mated to four virgin females of the



**Figure 1:** A, Sites along a latitudinal gradient in Chile where *Drosophila subobscura* flies were collected. Santiago and Puerto Montt are circled to indicate that these locations were employed to estimate the thermal niche available throughout the year. This is shown in B, where the potential role of behavioral thermoregulation to buffer environmental variation in temperature is plotted. Monthly mean and maximum temperatures ( $T_{a_{mn}}$  and  $T_{a_{max}}$ , respectively) for Santiago and Puerto Montt were employed to estimate the thermal niche available throughout the year (shaded areas; monthly minima were not employed because they represent nocturnal temperatures). We assume that flies selected those temperatures near their presumed optimal of 18°C (dashed line). This exercise shows that flies from these two locations can exhibit relatively similar body temperatures  $T_b$  (thick black lines), especially during the warm seasons between November and March. Whereas average differences in mean and maximum monthly temperatures between Santiago and Puerto Montt during these months correspond to 6.7° and 10.0°C, respectively, the average monthly difference in  $T_b$  estimated with this approach corresponds to 2.4°C ( $\Delta T = T_{\text{Santiago}} - T_{\text{Puerto Montt}}$ ). This suggests that behavioral thermoregulation, either by changing daily activity patterns or by selecting favorable microhabitats, could change considerably the thermal selection regime experienced by flies along the latitudinal gradient.

*ch-cu* marker strain to identify the gene arrangements of one set of the five major wild-type chromosomes (the other set of homologous chromosomes coming from the *ch-cu* strain; see Balanyà et al. 2004). The total number of isofemale lines employed to quantify inversion frequencies is given in table A1 (app. A).

From the complete set of isofemale lines per collection site, the  $F_1$  progeny from 50 randomly selected isofemale lines were used to set up one large outbred population in a Plexiglas cage (27 × 21 × 16 cm) maintained at 18°C (12L : 12D cycle) on a discrete-generation, controlled-larval-density regime, as described in Santos et al. (2004). After three generations, each population cage per site was split into three replicated cages kept on a 4-week, non-overlapping generation cycle at large census sizes (>1,500

breeding adults per cage). All fly handling was carried out at room temperature; CO<sub>2</sub> anesthesia was used only to sort virgin flies.

#### Thermal Preference Behavior in a Laboratory Thermal Gradient

At generation 5, eggs from all populations and replicated lines but that of Laja were collected and placed in 130-mL bottles at low density (~200 eggs per bottle containing 50 mL of food). In total, eight bottles per line were established, randomly assigned to two rearing temperatures with four bottles each: cold (13°C) and the presumed optimum temperature (18°C). Emerging adults were col-

lected daily and kept at the corresponding rearing temperature to be further assayed for  $T_p$ .

Thermal preferences were estimated following Rego et al. (2010) and Dolgova et al. (2010), with modifications. Briefly, flies were tested in a 60 × 31-cm (length × width) linear thermal gradient produced with an aluminum base plate with temperatures ranging from 3° to 37°C and constantly mapped at various positions along the plate with thermocouples. The aluminum plate was covered with Whatman's chromatography paper and a Plexiglas cover with 30 separate lanes, allowing individual flies to freely move along the gradient (i.e., any potential aggregation behavior was avoided; Dahanukar and Ray 2011). Adults were introduced and given 30 min to adjust, and then their positions were recorded four times at 10-min intervals. The median of the four measurements was used to estimate  $T_p$ . Taking into account the generation time of *D. subobscura* flies maintained at 13° or 18°C (~46 or 31 days, respectively; see Santos et al. 2004), the age of all flies tested was 9–10 days for flies that had developed at 13°C and 4–5 days for flies developed at 18°C. This minimized the difference in flies' physiological age between developmental temperatures.

Thermal preferences were recorded between 1000 and 1400 hours, which allowed the assaying of eight flies (four females and four males) from each replicated line of each population reared at a given temperature. Circadian variation was previously found to be important (Rego et al. 2010; but see Dolgova et al. 2010) and was randomized here by assigning two out of the eight flies (one male and one female) to each run. The full experiment was replicated five times during five consecutive days, so a perfectly balanced randomized block design was used. The complete data set is available in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.j939d> (Castañeda et al. 2013).

### Statistical Analyses

From an initial sample size of 1,200 flies (5 populations × 3 replicated lines × 2 sexes × 2 development temperatures × 5 days of measurements × 4 measurements per day), estimates of  $T_p$  were obtained for 1,190 flies. The linear mixed model used to analyze the thermal preferences for each sex was as follows:

$$T_{p(ijklmn)} = \mu + T_i + \wp_j + \Re(\wp)_{jk} + (T \times \wp)_{ij} + b_l + \beta x_m + e_{ijklmn}, \quad (1)$$

where  $\mu$  is the overall mean,  $T_i$  is the fixed effect of the  $i$ th developmental temperature (13°, 18°C),  $\wp_j$  is the random effect of the population (Santiago, Curicó, Chillán, Valdivia, or Puerto Montt),  $\Re(\wp)_{jk}$  is the random effect of the  $k$ th replicated line within population  $j$ ,  $b_l$  is the random effect

of the  $l$ th day when measurements were performed,  $\beta x_m$  is the effect explained by the covariate hour when the thermal preference was performed, and  $e_{ijklmn}$  is the residual error. This model was analyzed with restricted maximum likelihood estimation, using the "lmer" procedure in the "lme4" library (Bates et al. 2011) for R software, version 2.15 (R Development Core Team 2012). Random effects were tested by removing effects in a hierarchical fashion (i.e., moving from more complex interacting effects to single random effects) and comparing the models with and without a specific effect through a likelihood ratio test. In addition, a Markov chain Monte Carlo approach with 10,000 iterations was used to estimate the highest posterior density interval and  $P$  values associated with each fixed effect (Baayen 2008). These iterations were performed with the function "pvals.fnc" of the library "languageR" (Baayen 2011) for R software, version 2.15.

To test for latitudinal variation of thermal preferences, we performed separate ANCOVAs for females and males, including latitude as continuous predictor and developmental temperature (fixed effect) and replicated population (random effect) as categorical predictors. Linear regression was used to test for the association between frequency of chromosomal arrangements (arcsine transformed) and latitude.

Temperature data for each sampled locality were obtained from an online database published by the Dirección Meteorológica de Chile (<http://164.77.222.61/climatologia/>) from 1978 (the year when *D. subobscura* was first recorded at Puerto Montt; Brncic and Budnik 1980) to 2010 (when the actual samples were collected). From this database we obtained the annual mean and spring mean (average for September–November) temperatures at each sampled site. Also, monthly mean maximum and monthly mean temperatures for Santiago and Puerto Montt (i.e., the two extreme sampled sites) from 2007 to 2010 were employed to estimate the thermal niche available throughout the year (fig. 1B).

## Results

### Chromosomal Inversions

All 18 polymorphic chromosomal gene arrangements recorded in the sampled locations had been previously found in Chilean populations (table 1; their frequencies at each locality are given in table A1). Only three of them showed significant latitudinal slopes:  $A_{ST}$  increased in frequency with decreasing latitude, and the opposite pattern occurred for  $A_2$  and  $U_{ST}$ . However, this conclusion is (1) redundant, because inversion frequencies on a given chromosome are negatively correlated (i.e., a negative slope for  $A_{ST}$  implies a positive slope for  $A_2$ , since these are the only two arrangements segregating on chromosome A), and (2) biased, because no correction for multiple comparisons was

**Table 1:** Relationship between chromosomal arrangements and latitude in *Drosophila subobscura*

Chromosome arrangement	Sample size	Correlation	P
A <sub>ST</sub>	223	-.842 ± .146	.036*
A <sub>2</sub>	282	.827 ± .158	.042*
E <sub>ST</sub>	297	.216 ± .477	.681
E <sub>1+2</sub>	21	.453 ± .397	.366
E <sub>1+2+9</sub>	51	-.712 ± .247	.113
E <sub>1+2+9+12</sub>	83	.783 ± .193	.065
E <sub>1+2+9+3</sub>	91	-.690 ± .262	.129
J <sub>ST</sub>	171	.410 ± .416	.419
J <sub>1</sub>	376	-.403 ± .419	.429
O <sub>ST</sub>	138	-.347 ± .430	.500
O <sub>3+4</sub>	86	.089 ± .496	.867
O <sub>3+4+2</sub>	130	.261 ± .466	.618
O <sub>3+4+7</sub>	78	-.332 ± .445	.520
O <sub>3+4+8</sub>	88	.229 ± .474	.663
O <sub>5</sub>	23	.313 ± .451	.545
U <sub>ST</sub>	206	.912 ± .084	.011*
U <sub>1+2</sub>	204	-.742 ± .225	.092
U <sub>1+2+8</sub>	136	-.492 ± .379	.321

Note: Correlation coefficients (± standard error) between the frequencies of different polymorphic chromosomal arrangements (arcsine transformed) and latitude in *D. subobscura* flies collected in 2010 at several sites in Chile (see “Material and Methods”).

\* Significant correlation ( $P < .05$ ), not corrected for multiple comparisons (but see “Results”).

made. Focusing on the five standard gene arrangements (A<sub>ST</sub>, E<sub>ST</sub>, J<sub>ST</sub>, O<sub>ST</sub>, and U<sub>ST</sub>), which are typically cold-climate or high-latitude arrangements in the Old World (Menozi and Krimbas 1992), a sequential Bonferroni test (Rice 1989) suggests that no latitudinal clines for inversion polymorphisms remain in Chile.

The results in table 1 are at odds with former evolutionary time-series analysis in Chile (app. B). Thus, in previous surveys (1981, 1986, and 1999), all standard arrangements consistently increased in frequency with increasing latitude, in agreement with the Old World baseline, although statistical significance had been obtained only for E<sub>ST</sub> (all three surveys), O<sub>ST</sub> (1986 survey), and U<sub>ST</sub> (1986 survey; Balanyà et al. 2003).

#### Thermal Preferences

Average  $T_p$  (±SD) was  $18.4^\circ \pm 6.9^\circ\text{C}$  for females and  $17.9^\circ \pm 6.5^\circ\text{C}$  for males ( $F_{1,1188} = 1.39$ ,  $P = .243$ ), with “set-point” ranges ( $T_{\text{set}}$ : central 50% of records; Hertz et al. 1993) bounded by  $13.0^\circ$ – $24.5^\circ\text{C}$  and  $13.2^\circ$ – $24.0^\circ\text{C}$  (estimated from the whole data set), respectively. Importantly, the  $T_p$  of cold-developed flies was approximately  $2^\circ\text{C}$  higher than that of their counterparts developed at the presumed optimum temperature regardless of sex:

$19.4^\circ \pm 7.0^\circ\text{C}$  versus  $17.4^\circ \pm 6.6^\circ\text{C}$  for females reared at  $13^\circ$  and  $18^\circ\text{C}$ , and  $19.0^\circ \pm 6.6^\circ\text{C}$  versus  $16.9^\circ \pm 6.4^\circ\text{C}$  for males reared at  $13^\circ$  and  $18^\circ\text{C}$ , respectively.

Although interpopulation variation was not detected for  $T_p$  using the linear mixed model in equation (1) (table 2), the putative association of  $T_p$  with latitude can be better tested by linear regression analyses (Sokal and Rohlf 1995). Negative and parallel slopes were found for females developed at both rearing temperatures (fig. 2A), with a common slope  $b_{\text{female}} = -0.215 \pm 0.094$  (95% confidence interval:  $-0.400$  to  $-0.031$ ). On the other hand, the  $T_p$  of males did not significantly vary with latitude ( $b_{\text{male}} = -0.030 \pm 0.090$ ; 95% confidence interval:  $-0.206$  to  $0.146$ ; fig. 2B). The same qualitative result was obtained when  $T_p$  was regressed against the temperature data at each locality, because latitude is highly correlated with annual mean temperature ( $r = -0.996$ ,  $P < .001$ ) and spring mean temperature ( $r = -0.998$ ,  $P < .001$ ).

A short digression is in order here. Building on previous work by Anderson et al. (2007), Dillon et al. (2012) recently suggested that estimating  $T_p$  in a small ectotherm such as *Drosophila* requires knowledge of a null distribution of the position of flies in the thermal gradient. The null distribution is obtained from the individuals’ walking speed as a function of temperature ( $v(T)$ ). However, if we assume  $v(T)$  to be similar along the cline, then our conclusion that females’  $T_p$  shows a clinal pattern remains true because the null-model expectation would be the same across populations (see also Rego et al. 2010, p. 389). To sum up, the maximum difference in females’ average  $T_p$  among populations was  $2.5^\circ\text{C}$  when flies were reared at  $13^\circ\text{C}$  (Chillán vs. Puerto Montt) and  $1.8^\circ\text{C}$  when reared at  $18^\circ\text{C}$  (Curicó vs. Valdivia). These values clearly suggest that the absolute magnitude of evolutionary responses in  $T_p$  is comparable to its plastic response, owing to different rearing temperatures ( $1.9^\circ\text{C}$ ).

#### Discussion

In their comprehensive time-series analysis of inversion clines in New World populations of *Drosophila subobscura*, Balanyà et al. (2003) concluded (1) that these clines had not consistently continued to converge on the Old World baseline and (2) that the initial consistency of clinal evolutionary trajectories seen in the first surveys was no longer observed. Our results not only corroborate their findings but also point to a recent fading of inversion clines in Chile. Why has this happened?

One possible explanation is that the initial clinal patterns have been swamped by an increasing “random” migration among populations. Prevosti et al. (1985), in their original work, had already remarked that “differences in the rate of dispersal between individuals carrying different

**Table 2:** Mixed linear effect models for thermal preferences in females and males of *Drosophila subobscura*

Source	Variance component for random effects or coefficient for fixed effects (95% HPD)	Likelihood ratio test $\chi^2$	P
Females:			
Temperature	-1.957 (-3.085, -.912)	... <sup>a</sup>	.0001
Population	.250 (.000, 4.722)	.3679	.5441
Replicate nested in population	.000 (.000, .943)	.0000	1
Temperature $\times$ population	.000 (.000, .541)	.0003	.9871
Day (block effect)	.366 (.000, 5.174)	.7790	.3775
Covariate (plate hour)	1.520 (1.036, 1.994)	... <sup>a</sup>	.0001
Residuals	43.445 (38.616, 48.628)		
Males:			
Temperature	-2.088 (-3.150, -.993)	... <sup>a</sup>	.0010
Population	.000 (.000, 1.270)	.0000	.9994
Replicate nested in population	.000 (.000, 1.127)	.0000	1
Temperature $\times$ population	.132 (.000, 1.195)	.0000	1
Day (block effect)	.000 (.000, .777)	.0000	1
Covariate (plate hour)	1.199 (.756, 1.654)	... <sup>a</sup>	.0001
Residuals	40.026 (35.429, 44.624)		

Note: The *P* values of random effects are based on the likelihood ratio test and those of fixed effects on the highest posterior density (HPD) obtained from Markov chain Monte Carlo sampling.

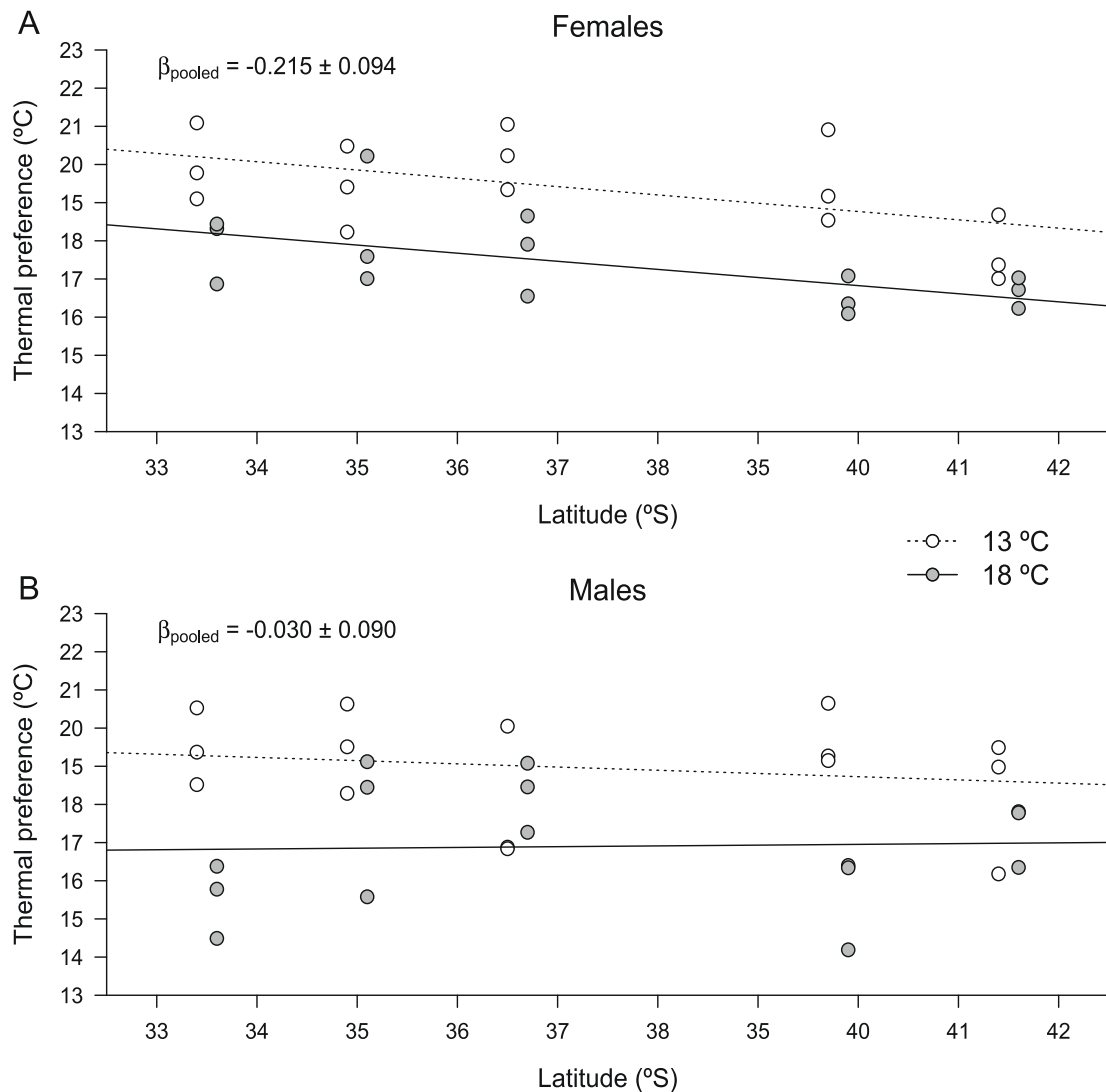
<sup>a</sup> Likelihood ratio tests were not performed for fixed effects.

chromosomal arrangements could also have contributed to the establishment of the clines" (p. 843; see also Balanyà et al. 2006). Some years later, Prevosti pointed out to one of us (M.S.) that their comment on inversion-dependent dispersal was tricky because bigger size could facilitate flight but was also associated with cold-climate gene arrangements (in a sample from Barcelona, northeastern Spain; Orengo and Prevosti 2002), and Puerto Montt (the likely site of the introduction) is one of the highest-latitude populations in Chile. We now know that the situation with body size is more complicated than this, because wing loading increases with decreasing temperatures in Chile (Gilchrist and Huey 2004), and that inversion-size associations in European and Chilean populations are generally different, probably because of the genetic bottleneck that occurred during the colonization (Fragata et al. 2010).

An additional possibility suggested by our results is that thermoregulatory behaviors might have attenuated environmental variation to such an extent that temperature-related traits are actually experiencing weak selection ("behavioral inertia," or the Bogert effect; Huey et al. 2003). Although figure 2A clearly shows that there are clinal genetic differences between populations in females'  $T_p$ , some caution is needed to correctly interpret these results. For instance, if flies thermoregulate behaviorally by selecting temperatures close to their thermal optimum during the warmest months, we expect that the mean difference in  $T_b$  between extreme sample sites would be approximately 2.4°C, whereas the average differences in mean and maximum mean temperatures are 6.7° and 10.0°C, respectively

(fig. 1B). Indeed, as the difference in annual mean (spring mean) temperature between the lowest- (Santiago: 14.7°C [14.8°C]) and the highest- (Puerto Montt: 10.0°C [9.5°C]) latitude populations is 4.7°C (5.3°C), basically the same difference we had between the two developmental temperatures assayed in this work (18° and 13°C), our results suggest that in natural conditions the  $T_p$  of the "average" female developed at Puerto Montt is expected to be about the same as the  $T_p$  of the "average" female developed at Santiago, because flies in Puerto Montt develop at lower temperatures than Santiago flies (fig. 2A). To put it differently, our results suggest that plastic responses of  $T_p$  to developmental temperature could offset (at least partially, because overlaps in ambient temperature are relatively small; fig. 1B) latitudinal genetic differences in this trait. Furthermore, plastic thermoregulation allows flies developed at colder temperatures to display a higher thermal preference, which could help compensate for the metabolic constraints imposed by low temperatures experienced during the development (Angilletta et al. 2010). This response is not exclusive to *D. subobscura*, because a negative relationship between rearing temperature and  $T_p$  has also been detected in *Drosophila melanogaster* and *Drosophila simulans*, where females reared at cooler temperatures have a higher  $T_p$  (Krstevska and Hoffmann 1994; but see Yamamoto and Ohba 1984).

It is nevertheless clear that these behavioral shifts are not fully compensatory. Otherwise, it would be difficult to explain the observed clinal pattern in  $T_p$ . Huey and Pascual (2009) have recently estimated seasonal variation of  $T_b$  in active *D. subobscura* flies (i.e., flies attracted to



**Figure 2:** Preferred temperatures in a laboratory thermal gradient for females (A) and males (B) from five populations of *Drosophila subobscura* distributed along a latitudinal gradient in Chile. White circles represent the average thermal preference of each replicated line for flies developed at 13°C and gray circles that for flies developed at 18°C. The slope of the average thermal preference against latitude is statistically significant for females but not for males.

baits with mashed bananas and yeast) from five North American populations spanning 12° of latitude. They found that mean field  $T_b$  varied from 8° to 29°C, well outside the set-point range estimated in the laboratory, and concluded that geographic shifts in ambient temperature may be a major evolutionary force, notwithstanding the thermoregulatory behavior of *D. subobscura* flies. However, it is not clear from their paper whether the time of maximum activity of flies was the same for both sexes, which are known to be differentially attracted to banana baits according to the season and the hour of collection

(Argemí 2002; F. Rodríguez-Trelles, personal communication). This is important because latitudinal variation in  $T_p$  is not consistent between sexes, as a significant slope was detected only in females (fig. 2), which agrees with our previous result of low repeatability of  $T_p$  in males (Rego et al. 2010). Therefore, males' activity times may be less driven by thermoregulatory concerns than those of females; for example, mate-seeking behavior is expected to be more important in males, as their reproductive success is determined by the number of matings achieved, which seems to be constant across wide temperature ranges

(Schnebel and Grossfield 1984). Active thermoregulation in females may ultimately determine the thermal environment experienced by eggs and larvae, which, given the negative relationship between  $T_p$  and developmental temperature, might explain how flies minimize the effect of seasonal thermal fluctuations across generations. Future experiments to test whether or not oviposition site behavior of *Drosophila* females is indeed related to their thermal preferences are badly needed. To our knowledge, Jones et al. (1987) performed the only field study aimed at estimating *D. melanogaster*'s thermal niche. They released and recaptured a temperature-sensitive eye color mutant at two localities, one lowland and the other highland, with a difference of 4.5°C in mean air temperature and found that the average difference in development temperature of offspring flies between localities was only 1.3°C. Jones et al. (1987) interpreted this result as evidence of behavioral thermoregulation but did not relate females' oviposition thermal preferences to the temperatures experienced by their offspring (Feder et al. 1997b, p. 586).

To conclude, clinal variation in  $T_p$  suggests that thermal

selection partly explains the observed genetic differences between populations. However, plastic behavioral thermoregulation may now be buffering latitudinal differences in environmental temperatures to such an extent that little selection for traits at least partially linked to thermal effects (e.g., inversions) is already taking place. An important exception may involve temperature extremes (Feder et al. 1997a; Hoffmann 2010), which could be the real selective agent underlying chromosomal inversion clines in *D. subobscura* (Rego et al. 2010; Calabria et al. 2012; see also Huey and Pascual 2009). Interestingly, of the three distribution areas of *D. subobscura* included in the time-series analysis of inversion clines (fig. 1 in Prevosti et al. 1988), seasonal variation in temperature is much lower in South America than in the Northern Hemisphere (Chown et al. 2004), and extreme thermal events have been recorded in the Palearctic region and in North America but are not so frequent in South America (Alexander et al. 2006). It would be fascinating to extend this work to North American flies and see whether the original inversion clines still remain.

## APPENDIX A

### Chromosomal-Arrangement Frequencies

**Table A1:** Chromosomal-arrangement frequencies of *Drosophila subobscura* along a latitudinal gradient in Chile

Chromosomal arrangement	Santiago 33°27'S	Curicó 34°58'S	Chillán 36°30'S	Laja 37°12'S	Valdivia 39°48'S	Puerto Montt 41°28'S
A <sub>ST</sub>	52.4 (43)	42.7 (38)	48.5 (50)	47.7 (51)	40.6 (26)	24.6 (15)
A <sub>2</sub>	47.6 (39)	57.3 (51)	50.5 (52)	52.3 (56)	59.4 (38)	75.4 (46)
A <sub>new</sub>	.0 (0)	.0 (0)	1.0 (1)	.0 (0)	.0 (0)	.0 (0)
E <sub>ST</sub>	49.5 (45)	51.0 (53)	60.2 (65)	58.4 (66)	56.1 (37)	51.6 (33)
E <sub>1+2</sub>	4.4 (4)	4.8 (5)	1.9 (2)	1.8 (2)	6.1 (4)	6.3 (4)
E <sub>1+2+9</sub>	13.2 (12)	12.5 (13)	5.6 (6)	9.7 (11)	6.1 (4)	7.8 (5)
E <sub>1+2+9+3</sub>	18.7 (17)	16.3 (17)	16.7 (18)	15.9 (18)	16.7 (11)	15.6 (10)
E <sub>1+2+9+12</sub>	13.2 (12)	15.4 (16)	15.7 (17)	14.2 (16)	15.2 (10)	18.8 (12)
E <sub>new</sub>	1.1 (1)	.0 (0)	.0 (0)	.0 (0)	.0 (0)	.0 (0)
J <sub>ST</sub>	31.9 (29)	30.8 (32)	31.2 (34)	30.1 (34)	30.3 (20)	34.4 (22)
J <sub>1</sub>	68.1 (62)	69.2 (72)	68.8 (75)	69.9 (79)	69.7 (46)	65.6 (42)
O <sub>ST</sub>	31.9 (29)	21.9 (23)	29.4 (32)	22.1 (25)	16.9 (11)	28.1 (18)
O <sub>2</sub>	1.1 (1)	.0 (0)	.0 (0)	.0 (0)	.0 (0)	.0 (0)
O <sub>3+4</sub>	22.0 (20)	11.4 (12)	14.7 (16)	13.3 (15)	15.4 (10)	20.3 (13)
O <sub>3+4+2</sub>	20.9 (19)	25.7 (27)	18.3 (20)	28.3 (32)	26.2 (17)	23.4 (15)
O <sub>3+4+7</sub>	9.9 (9)	17.1 (18)	17.4 (19)	15.9 (18)	12.3 (8)	9.4 (6)
O <sub>3+4+8</sub>	13.2 (12)	21.9 (23)	11.9 (13)	13.3 (15)	23.1 (15)	15.6 (10)
O <sub>3+4+8+2</sub>	.0 (0)	.0 (0)	.9 (1)	.0 (0)	.0 (0)	.0 (0)
O <sub>3+4+8+7</sub>	.0 (0)	.0 (0)	.0 (0)	.0 (0)	1.5 (1)	.0 (0)
O <sub>5</sub>	1.1 (1)	1.0 (1)	7.3 (8)	7.1 (8)	4.6 (3)	3.1 (2)
O <sub>7</sub>	.0 (0)	1.0 (1)	.0 (0)	.0 (0)	.0 (0)	.0 (0)
U <sub>ST</sub>	30.8 (28)	29.5 (31)	40.4 (44)	34.5 (39)	50.0 (33)	48.4 (31)
U <sub>1+2</sub>	46.2 (42)	38.1 (40)	33.9 (37)	42.5 (48)	24.2 (16)	32.8 (21)
U <sub>1+2+8</sub>	23.1 (21)	32.4 (34)	24.8 (27)	22.1 (25)	25.8 (17)	18.8 (12)
U <sub>dup</sub>	.0 (0)	.0 (0)	.9 (1)	.9 (1)	.0 (0)	.0 (0)

Note: Chromosomal-arrangement frequencies (in %; counts are given in parentheses) of the six populations of *D. subobscura* sampled in Chile in spring 2010.

## APPENDIX B

Historical Chromosomal Inversion Clines of *Drosophila subobscura* along a Latitudinal Gradient in Chile

What is the evidence that chromosomal inversion clines have faded in Chile? To address this question, we collected the data previously published by Prevosti et al. (1985) and Balanyà et al. (2003), which include the first study of chromosomal inversion polymorphism in Chile in 1981, when latitudinal clines had already been detected, and the last study in 1999, which is most relevant for comparisons with our present survey. The various gene arrangements of *D. subobscura* can be divided into two groups based on the correlation of gene arrangement frequencies and geographic latitude in native Palearctic populations (Menozzi and Krimbas 1992; Balanyà et al. 2003): “cold-climate” arrangements, with a positive correlation ( $A_{ST}$ ,  $E_{ST}$ ,  $J_{ST}$ ,  $O_{ST}$ ,  $O_5$ ,  $U_{ST}$ ), and “warm-climate” arrangements, with a negative correlation ( $A_2$ ,  $E_{1+2}$ ,  $E_{1+2+9}$ ,  $E_{1+2+9+12}$ ,  $E_{1+2+9+3}$ ,  $J_1$ ,  $O_{3+4}$ ,  $O_{3+4+8}$ ,  $O_{3+4+7}$ ,  $O_{3+4+2}$ ,  $U_{1+2}$ ,  $U_{1+2+8}$ ). Regardless of the statistical significance of the frequency-latitude correlations, Prevosti et al. (1985) were also concerned with agreement in the signs of the correlations between the native Old World and the colonizing New World populations.

Table B1 summarizes this information for these historical collections and also includes our recent sample. A striking trend discussed by Prevosti et al. (1985) was that in just 3 years after *D. subobscura* arrived in Chile, all gene arrangements but one showed a correlation with latitude whose sign matched that found in the Old World. This trend was also maintained in the last historical sample (1999). However, in our recent 2010 survey, 10 signs agree and 8 disagree with the Old World baseline ( $P = .407$ ; one-tailed sign test). The disagreement basically comes from the gene arrangements on chromosomes A, E, and O. Interestingly, Rego et al. (2010) found that gene arrangements on chromosomes A and O are related to thermal preferences in a laboratory thermal gradient and that gene arrangements on chromosome E are related to flies’ heat knockdown resistance. However, Rego et al. (2010) used flies from a European population (Adraga, Portugal), and their results cannot be extrapolated to South American populations because the association between phenotypic traits and inversions can have changed because of the bottleneck effect that occurred during colonization (see Fragata et al. 2010).

A problem with the sign test is that gene arrangement frequencies on the same chromosome are not statistically independent, and Prevosti et al. (1985) did not correct for this. Focusing only on the cold-climate standard gene arrangements on the five independently segregating chromosomes, there was a perfect agreement in the sign of the five correlations in the Old and New World in 1981 and

1999 ( $P = .031$ ; one-tailed sign test). However, in our 2010 survey, three signs agree and two disagree ( $P = .500$ ; one-tailed sign test).

Table B1: Historical chromosomal inversion clines of *Drosophila subobscura* in Chile

Chromosomal arrangement	1981	1999		2010
		A	B	
Cold climate:				
A <sub>ST</sub>	+	+	+	—*
E <sub>ST</sub>	+**	+	+	+
J <sub>ST</sub>	+	+	+	+
O <sub>ST</sub>	+	+	+	—
O <sub>5</sub>	+*	+	+	+
U <sub>ST</sub>	+*	+	+	+*
Warm climate:				
A <sub>2</sub>	—	—	—	+*
E <sub>1+2</sub>	—	+	+*	+
E <sub>1+2+9</sub>	—\$	—*	—	—
E <sub>1+2+9+12</sub>	—	—*	—\$	+
E <sub>1+2+9+3</sub>	—	—\$	—	—
J <sub>1</sub>	—	—	—	—
O <sub>3+4</sub>	—	+	—	+
O <sub>3+4+8</sub>	+	+	+	+
O <sub>3+4+7</sub>	—\$	—	+	—
O <sub>3+4+2</sub>	—*	—**	—*	+
U <sub>1+2</sub>	—\$	—	—	—
U <sub>1+2+8</sub>	—	—	—	—

Note: The sign of the correlation (with its statistical significance) between chromosomal-arrangement frequencies (arcsine transformed) and geographic latitude for the first (1981) and last (1999) historical samples collected in Chile, with those from our survey in 2010. In the Old World baseline, the sign of the correlation with latitude is positive for cold-climate and negative for warm-climate gene arrangements. The signs of correlations in 1999 are for the whole data set (A; agreement with the Old World baseline:  $P = .004$ , one-tailed sign test) and after excluding Coyahique ( $45^{\circ}35'S$ ; B;  $P = .004$ , one-tailed sign test); this high-latitude population was not included in our 2010 survey because very few flies were collected.

\$  $P < .10$ .

\*  $P < .05$ .

\*\*  $P < .01$ .

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