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CLINAL PATTERNS OF CHROMOSOMAL INVERSION POLYMORPHISMS IN *DROSOPHILA SUBOBSCURA* ARE PARTLY ASSOCIATED WITH THERMAL PREFERENCES AND HEAT STRESS RESISTANCE

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Latitudinal clines in the frequency of various chromosomal inversions are well documented in *Drosophila subobscura*. Because these clines are roughly parallel on three continents, they have undoubtedly evolved by natural selection. Here, we address whether individuals carrying different chromosomal arrangements also vary in their thermal preferences (T_p) and heat stress tolerance (T_{ko}). Our results show that although T_p and T_{ko} were uncorrelated, flies carrying "cold-adapted" gene arrangements tended to choose lower temperatures in the laboratory or had a lower heat stress tolerance, in line with what could be expected from the natural patterns. Different chromosomes were mainly responsible for the underlying genetic variation in both traits, which explains why they are linearly independent. Assuming T_p corresponds closely with temperatures that maximize fitness our results are consistent with previous laboratory natural selection experiments showing that thermal optimum diverged among thermal lines, and that chromosomes correlated with T_p differences responded to selection as predicted here. Also consistent with data from the regular tracking of the inversion polymorphism since the colonization of the Americas by *D. subobscura*, we tentatively conclude that selection on tolerance to thermal extremes is more important in the evolution and dynamics of clinal patterns than the relatively "minor" adjustments from behavioral thermoregulation.

KEY WORDS: **Adaptation, chromosomal polymorphisms, latitudinal variation, stress tolerance, thermoregulation.**

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Clinal patterns can reflect variable selection in space and are thought to be maintained by a trade-off between dispersal and local adaptation. Although clines by themselves are not sufficient to infer adaptive divergence, strong evidence of selection is obtained if those patterns can be independently replicated and understood in terms of likely fitness effects (Endler 1977). This approach has been used effectively with the invasive species *Drosophila subobscura* ever since its first appearance in South (Puerto Montt, Chile, 1978; Brncic and Budnik 1980) and North America (Port Townsend, WA, 1982; Beckenbach and Prevosti 1986).

An early comprehensive summary of the wealthy inversion polymorphism of *D. subobscura* revealed that the complex gene arrangements (with overlapping and nonoverlapping inversions) from more equatorial Palearctic populations are gradually replaced by the so-called standard gene arrangements in the five major acrocentric chromosomes as populations approach high latitudes (Krimbas and Loukas 1980). Disentangling adaptive explanations from purely historical processes that could generate this pattern (i.e., northward migration of the populations of *D. subobscura* after the last glacial period) was only possible when Prevosti et al. (1985) described rapid convergent evolution in the signs of the correlations between gene arrangements and latitude a few years following the American invasion. Selection must have to be strong and consistent along the latitudinal gradients to explain the rapid emergence of independent parallel clines (summarized in Balanyà et al. 2003) in such a highly dispersive species as *D. subobscura* (Ayala et al. 1989). Yet, not much notice was taken of the final remarks in Prevosti's et al. original work (but see Balanyà et al. 2006): "Differences in the rate of dispersal between individuals carrying different chromosomal arrangements could also have contributed to the establishment of the clines." This point is important and recent theoretical work shows that model assumptions with random diffusive dispersal or fitness-dependent dispersal can have important implications in the structure and dynamics of clines (Armsworth and Roughgarden 2008).

The simple idea that environmental latitudinal gradients favor different inversions at different latitudes remains attractive, and several lines of evidence suggest that temperature may be the underlying selective factor. First, a long time-series experiment starting in 1976 at a northwestern Spanish population showed that the seasonal climatic cycle induces seasonal changes in inversion frequencies on chromosome O that are consistent with their latitudinal patterns (Fontdevila et al. 1983; Rodríguez-Trelles et al. 1996). Second, long-term trends were superimposed on the seasonal cycles with "southern," low-latitude inversions increasing in frequency thus suggesting a directional response to current climate warming (Rodríguez-Trelles et al. 1996; Rodríguez-Trelles and Rodríguez 1998). More recent and comprehensive studies have shown that the genetic constitution of *D. subobscura* populations worldwide is indeed responding to climate change (Balanyà et al. 2006). However, it is not yet clear whether the shifts in inversion frequencies represent local selection, confound long-term trends with the presence of a shifting seasonal component (c.f. Rodríguez-Trelles and Rodríguez 2007; Balanyà et al. 2007), or simply reflect an invasion from more equatorial populations (see Santos 2007).

The putative role of temperature per se in the formation of inversion clines has been tested by letting replicated lines of *D. subobscura* evolve in the laboratory at different constant temperatures (Santos et al. 2005). In this experiment gene arrangements on all chromosomes generally shifted in ways inconsistent with expectations based on clinal patterns. It is nevertheless obvious that keeping flies in isolated populations under different selective regimes misses the multitude of additional factors that complicate the simplistic view in this kind of experiments: that natural selection can be embodied in its totality in laboratory population cages. Ectotherms in the wild use thermoregulatory behaviors to avoid—or at least reduce—the impact of thermal fluctuations and can maintain body temperature (T_b) within relatively narrow boundaries (e.g., by modifying daily activity patterns and selecting favorable microclimates; Stevenson 1985). Behavioral thermoregulation in *D. subobscura* could ameliorate effects of seasonal and latitudinal variation in air temperature, hence the actual selective pressures implicated in the rapid clinal evolution of chromosomal inversions might be weaker than originally thought (Huey et al. 2003) and could even conflict with those in the laboratory (e.g., by forcing flies that would otherwise choose colder or warmer settings to compete at fixed constant temperatures).

Seasonal variation of T_b in active *D. subobscura* flies from five North American populations spanning 12° latitude has been recently estimated by Huey and Pascual (2009). To evaluate if these flies can be effective thermoregulators (Hertz et al. 1993) they also measured their thermal preferences (T_p: the body temperature an organism chooses when provided with a range of potential temperatures; see Dillon et al. 2009) using a laboratory thermal gradient. The results show that mean field T_b varied from 8°C to 29°C, well outside the relatively narrow set-point range (central 50% of preferred body temperatures: 21.2°C – 25.9°C). Therefore, although *D. subobscura* flies can behaviorally thermoregulate, geographic shifts in ambient temperature may nevertheless be a major evolutionary force in generating the clinal patterns. A corollary of their work is to question the validity of the aforementioned laboratory thermal selection experiment because the imposed thermal regimes might not match the optimum temperatures of the various gene arrangements (Fig. 1).

Here, we explore whether the thermoregulatory behavior and thermal tolerance of *D. subobscura* have a genetic component. More specifically, we address whether individuals carrying different chromosomal arrangements also vary in their T_p and heat tolerance (T_{ko}: the temperature required to knock out a fly

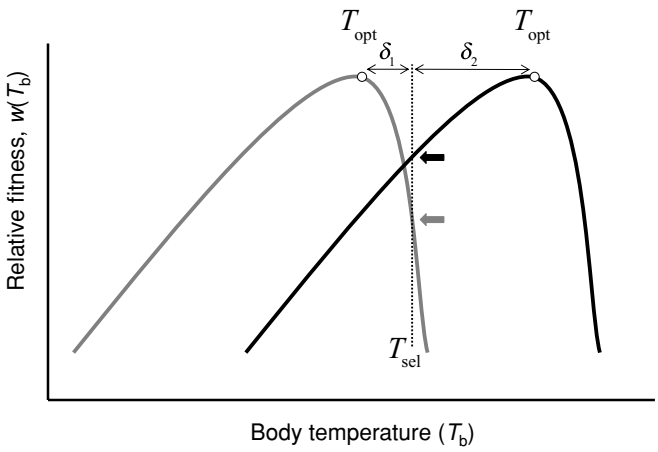

Figure 1. Relative fitness $w(T_b)$ as a function of T_b for two hypothetical genotypes under a constant thermal regime (T_{sel}; doted line) in a laboratory natural selection experiment. The gray line is the thermal fitness curve of the cold-adapted genotype, which rises gradually to an optimum temperature (T_{opt}; white dot) and then drops rapidly (Huey and Stevenson 1979). The imposed thermal selection regime could be labeled "cold" because $|\delta_1| < |\delta_2|$ ($|\delta_i|$ is the absolute difference $|T_{opt} - T_{sel}|$). However, the asymmetry of T_b fitness curves (left-skewed) results in a higher fitness of the warm-adapted genotype (black arrow above grey arrow). The thermal selection lines set up by Santos et al. (2005) were allocated to three different temperatures based on previous information: 13°C (cold), 18°C (the presumed optimum), and 22°C (warm). These characterizations are clearly challenged if the preferred temperature of *D. subobscura* is in the range 21.2°C–25.9°C (Huey and Pascual 2009), but our present estimates are substantially lower (see Results). The important point here is that our ignorance of the thermal dependence of fitness might cause apparent conflicts between nature and laboratory; that is, a lack of correspondence between the outcomes from laboratory thermal selection and clinal patterns could simply be a "fault" in the experiment due to a mismatch between the thermal regimes and the actual optimum temperatures of the various gene arrangements.

in a water bath). Our analysis was motivated by the hypothesis that inversion clines are indeed driven by thermal selection (population cage results notwithstanding; Fig. 1), and the idea that genetic variation in thermoregulatory abilities might underlie the evolution of clines of chromosomal arrangements. Some evidence indicates that diurnal activity patterns can vary according to gene arrangements in this species (Savkovic et al. 2004), which suggests that fitness-dependent dispersal (as pointed out by Prevosti et al. 1985) due to different physiological optimal temperatures of the various inversion carriers could be at work in natural populations. Furthermore, because *D. subobscura* can occasionally experience injurious or lethal effects of very high T_b's—for example, larval feeding patches could become lethally hot (Feder et al. 1997), and the former maximum of 29°C for active flies in the field would certainly be considered as quite stressful by most researchers working with this species (see Krimbas 1993)—upper thermal tolerance likely imposes critical limits on fitness. Laboratory studies with *D. melanogaster* have associated heat-tolerance variation with genetic variation at candidate loci for thermoresistance linked to chromosomal inversions (e.g., Anderson et al. 2003; Hoffmann and Weeks 2007), and a larger than expected number of genes putatively involved in thermal adaptation in *D. subobscura* map inside inverted chromosomal segments (Laayouni et al. 2007). Our results show that flies carrying "cold-adapted" gene arrangements (typically "high-latitude" arrangements; see Statistical Methods below) tend to choose lower temperatures in the laboratory or have a lower heat stress tolerance, in line with what could be expected from the natural patterns. Different chromosomes are mainly responsible for the underlying genetic variation in both traits, which also explains why thermal preference and thermal tolerance were linearly independent.

Materials and Methods

POPULATION STOCKS AND SAMPLING PROTOCOL

The *D. subobscura* stocks originated from 210 wild females collected by one of us (CR) in a pinewood near Adraga (Portugal; 38°47′N, 9°28′W) in April 2008. From their offspring a large outbred population was set up in a plexiglas cage (27 × 21 × 16 cm^3) and maintained at 18°C (12L : 12D) on a discrete generation, controlled larval crowded regime as described in Santos et al. (2004). After three generations the population was split into three replicated lines kept on a four-week, nonoverlapping generation cycle at large population sizes (>1500 breeding adults per population). At generation eight, eggs were sampled from each line and placed in vials under uncrowded conditions (35 eggs/vial with 6 mL of food) to obtain the experimental flies. Our aim was to evaluate thermal preference (T_p) and knockout temperature (T_{ko}) for each individual fly, and determine whether there is a link between these variables and polymorphic chromosomal inversions that exhibit latitudinal clines on three continents. Sample size requirements for a genetic study, such as this, are usually quite high, and it was therefore necessary to sample eggs from the replicated lines once a week during four weeks to appropriately handle the amount of work involved (i.e., measuring over 1000 individual flies; see below). To minimize ageing effects of the parental flies from which the eggs were obtained young adults (∼ one-week old) were dumped twice in the collecting cages: prior to the beginning of the egg-sampling period and two weeks later. Virgin females and males were separated not less than 6 h after eclosion, and maintained in bottles with controlled adult densities. All fly handling was done at room temperature and CO_2 anesthesia was only used to collect virgin flies.

CHROMOSOMAL INVERSIONS

Virgin flies were subsequently collected from the bottles and individually crossed after three days to three or four virgin flies from the *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). The wild-type flies were derived from the eggs sampled along four weeks as indicated above, and a total of 1320 crosses (660 females and 660 males) were split into 11 batches with 120 crosses each (20 females and 20 males per line × three replicated lines): three batches per week in the first three weeks and two batches in the last week. The flies were let to mate for one week, after which the wild-type flies were removed to measure their thermal preferences. The vials used for the crosses were kept until gene arrangements were identified from inspection of salivary gland squashes of one F_1 female's third-instar larva (see e.g., Santos et al. 2005).

THERMAL PREFERENCE BEHAVIOR IN A LABORATORY GRADIENT

To assay for thermal preference, a linear thermal gradient ranging from 11°C to 29°C was produced with an aluminum block (30 cm length × 31 cm width × 2.5 cm height) resting on a hot and cold plate at each end (see Sayeed and Benzer 1996). Temperatures along the gradient were measured on the aluminum block with thermocouples and were reproducible through the experiment. The range of temperatures achieved is encountered by active flies in the field (Huey and Pascual 2009). A plexiglas cover with 30 separate lanes was placed on the block, creating suitable spaces for individual flies to freely move along the aluminum base. The relative humidity along the lanes was not measured, but condensation was not a problem in the experiment. The plexiglas plate was lightly dusted with quinine sulfate powder (a repellant for *Drosophila*; Quinn et al. 1974) to prevent flies from escaping the temperature gradient by resting on the walls or roof of the lane. Adult flies (15 females and 15 males) were gently aspirated from the vials, introduced into the lanes at room temperature (22°C–23°C) and given ∼30 min to adjust. Afterwards the aluminum base was placed on the plates to generate the thermal gradient (∼10 min) and each fly's position was recorded four times every 10 min: from 40 to 70 min (counting from the time when the thermal gradient was applied). To minimize circadian variations, four trials were run between 1200 h and 1800 h, which allowed assaying all flies (60 females and 60 males) from a batch of crosses on the same day. We used the median of the four measurements to estimate T_p of each fly. Measurements were performed in a room with a constant temperature, and the flies were assayed under white light illumination. The age of all flies tested was synchronized at ∼10 days post-imaginal eclosion. After the thermal preference assay, each fly was gently removed from the lane and individually placed in a vial with fresh food at 18°C for the subsequent assay of heat stress tolerance (below).

Repeatability of thermal preference was estimated from an independent sample of 90 flies (45 females and 45 males) as follows. We first recorded each fly's position without a thermal gradient every 10 min for 40 min. Subsequently, we generated the thermal gradient and measured T_p as described above. After the assays the flies were individually placed in vials with fresh food at 18°C, and the whole experimental protocol was repeated on the next day. From the two T_p estimates for each individual, repeatability was estimated as the intraclass correlation coefficient (Sokal and Rohlf 1995, p. 213; Falconer and Mackay 1996, p. 136).

HEAT RESISTANCE

One day after measurements of thermal preference, flies were assayed for heat resistance. Measurements were performed at ∼1400 h; that is, after 20–26 h from the time when their thermal preferences were recorded. Flies were individually placed in sealed empty vials and immersed in two water baths (60 flies per water-bath) at $T_{min} = 24$°C. Every 10 min individuals were scored for mobility (fly active or knocked out) and the temperature of the water was increased by $\Delta T = +1$°C (it took ∼2–3 min for the water bath to reach equilibrium). The procedure was repeated until the water baths reached T_{max}, defined as the temperature when the last active fly was knocked out ($T_{max} = 38$°C was the upper limit in the assays; median $T_{max} = 37$°C). For each fly T_{ko} was estimated as the temperature taken to knock it out.

STATISTICAL METHODS

Different people were involved in collecting data for T_p, T_{ko}, and chromosomal inversions, which guaranteed a blind experiment. From an initial sample size of 1320 individuals, we measured T_p in 1215 flies (614 females and 601 males), T_{ko} in 1195 (606 females and 589 males), and identified chromosomal inversions of 1119 individuals (559 females and 560 males; in a few cases not all gene arrangements from the wild-type chromosomal set could be identified). Based on the experimental design, the biometric effect of chromosomal inversions on thermal preference can be readily analyzed as the linear model

$$T_{p(ijklm)} = \mu + \Re_i + A_j + S_k + AS_{jk} + \beta x_l + e_{ijklm}, \qquad (1)$$

where μ is the overall grand mean, $\Re_i$ is the random effect of the ith batch ($i = 1, 2, \ldots, 11$), A_j is the fixed effect of the gene arrangement for a particular chromosome ($j = 1, 2$; see below), S_k is the fixed effect of sex, βx_l is the effect explained by the covariate hour when the temperature preference assay was performed, and e_{ijklm} is the residual error associated with the thermal preference (T_p) of the mth fly from the kth sex with the jth gene arrangement that was assayed in the ith batch at the lth hour. Likewise, the linear

model used to analyze the effect of chromosomal inversions on knockout temperature was

$$T_{\mathrm{ko}(ijklm)} = \mu + \Re_i + A_j + S_k + AS_{jk} + \omega_l + e_{ijklm}, \qquad (2)$$

where ω_l is the random effect of the lth ($l = 1, 2$) water bath (ω_l can instead be introduced as a covariate and the results are the same). Variation among the three replicated lines was negligible (results not shown) and, therefore, we have conveniently grouped flies across lines. A type III Sum of Squares was used. Note that in linear models (1)–(2) all interactions with batches are assumed to be part of the experimental error. Preliminary tests (results not shown) indicated that the pooling was justified (see Winer 1971, pp. 391–394).

The various gene arrangements in the dataset (Appendix 1) can be divided into two groups based on the study by Menozzi and Krimbas (1992) in Palearctic populations. Group 1 ("cold-adapted") includes all gene arrangements that show a negative correlation coefficient with maximum temperatures along the cline, or a positive correlation coefficient with latitude: A_{st}, A_1, J_{st}, U_{st}, E_{st} and O_{st}. Conversely, group 2 includes "warm-adapted" gene arrangements: A_2, A_{2+6}, J_1, U_{1+2}, U_{1+2+8}, E_{1+2+9}, $E_{1+2+9+3}$, $E_{1+2+9+12}$, O_{3+4}, O_{3+4+1} and O_{3+4+8}.

To establish the statistical significance of the gene arrangement main effect (cold vs. warm adapted) we also conducted permutation tests (Edgington 1995; Manly 1997) from the residuals of linear equations (1) and (2) with all effects included but gene arrangement. Permutation tests are far less sensitive to the presence of outliers and are particularly necessary with unequal sample sizes, as was always the case here. The computer programs used for statistical data analyses were MATLAB 7.1 (MathWorks, Natick, MA) together with the collection of tools supplied by the Statistics Toolbox, and the statistical software packages STATISTICA 8.0 (StatSoft, Tulsa, OK) and SPSS 15.0 (SPSS, Chicago, IL).

Results

THERMAL PREFERENCE IN THE LABORATORY

Repeatability

In the absence of a temperature gradient (aluminum block fixed at 22°C–23°C) the median positions of flies were scattered on the aluminum base and uncorrelated between the two days (Spearman $r_S = -0.131, P > 0.05$), but significantly deviated from a uniform distribution (Kolmogorov-Smirnov $D = 0.380$, $P = 0.020$; see inset plot in Fig. 2). The rate of movement, estimated as the distance (mean ± SD) traveled by the flies between observations was 19.08 ± 18.49 cm for females and 15.19 ± 16.27 cm for males. The results therefore suggest that the ~30 min given to the flies to adjust before the thermal gradient was applied (see above) were enough to generate an approximate null distribution along the aluminum base (Anderson et al. 2007).

Individual differences in median thermal preferences (T_p) were significant repeatable for females (intraclass correlation $r_I = 0.254$; $F = 1.68$, df = 44, 45, $P = 0.043$) but not for

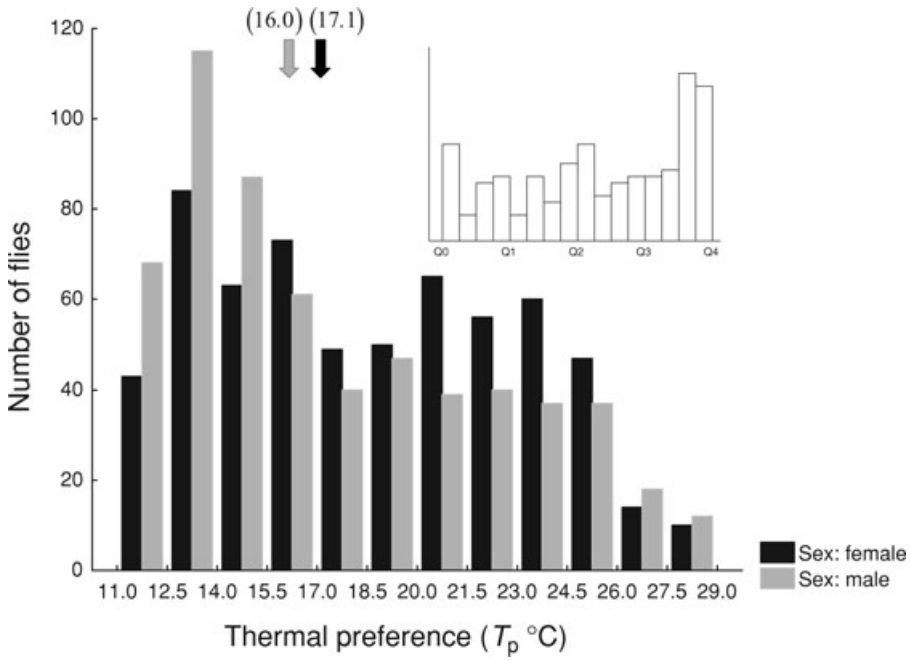

Figure 2. Distribution of *D. subobscura* on the temperature gradient grouped in intervals of 1.5°C. The inset plot shows the roughly even distribution of flies (total number of 45 females and 45 males assayed on two days) when 22°C–23°C was sustained across the block (Q# stands for the quantile distance). Arrows point to the average thermal preference for each sex.

males ($r_I = 0.054$; $F = 1.11$, df = 44, 44, $P = 0.362$; one male flew away before the assay on the second day). We can therefore conclude that, at least for females, estimates of T_p is the reliable assessment of their thermal preferences.

Preferred temperatures

The temperature preference responses of the flies are plotted in Figure 2. Individuals clearly moved toward the colder region of the thermal gradient, and the distribution of their resting positions on the aluminum base was noticeably different from that obtained without gradient (Kolmogorov–Smirnov $D = 0.706$, $P < 0.001$; females and males pooled). An analysis of covariance (ANCOVA) using sex as categorical predictor (after correcting for batch effects) and hour of the thermal preference assay as a covariate detected a highly significant difference between females and males ($F = 17.08$, df = 1, 1202, $P < 0.001$). Average (±SD) T_p was 17.1 ± 4.6°C for females and 16.0 ± 4.8°C for males (Levene's test for homogeneity of variances: $F = 0.54$, $P = 0.462$). The central 50% of records were bounded by 13.1–20.8°C for females and 12.3–19.8°C for males, which are the preferred temperature ranges or "set point" ranges (T_{set}; Hertz et al. 1993).

HEAT RESISTANCE

The distributions of knockout temperatures are plotted in Figure 3. An analysis of variance (ANOVA) using sex as categorical predictor (after correcting for batch effects and water bath) detected a highly significant difference between females and males ($F = 45.52$, df = 1, 1182, $P < 0.001$). Average (±SD) T_{ko} was 34.7 ± 1.3°C for females and 34.1 ± 1.6°C for males (Levene's $F = 23.07$, $P < 0.001$). Permutation tests (with 10,000 random permutations after correcting for batch effects and water bath) confirmed that T_{ko} was higher for females ($P = 0.0001$).

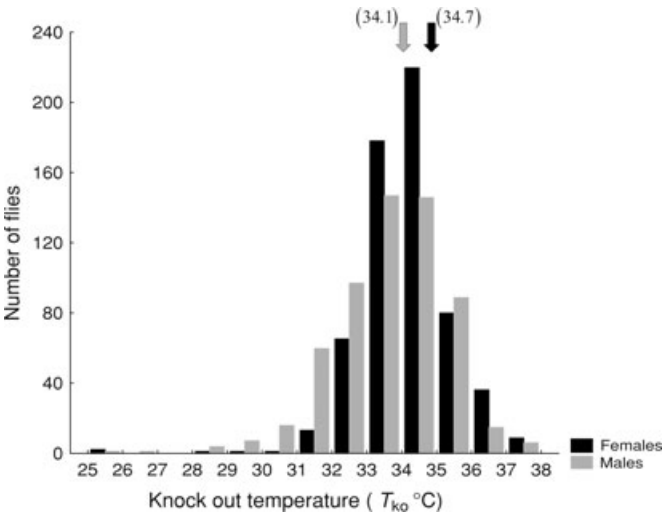

Figure 3. Distribution of knockout temperatures by increasing $\Delta T = +1$°C from $T_{min} = 24$°C to $T_{max} = 38$°C in the water baths. Arrows point to the average T_{ko} for each sex.

The relationship between T_p and T_{ko}, estimated from the partial correlation coefficient holding constant the variables sex, batch, hour of the thermal preference assay and water-bath, was not significant ($r_{T_p \cdot T_{ko}} = -0.002$; $t = 0.06$, df = 1189, $P = 0.956$). It is clear that thermal preference and knockout temperatures are nearly orthogonal to each other. In addition, these results suggest that there was no induced thermotolerance; that is, those flies that happened to choose a higher T_p did not show a higher T_{ko} on the next day. In summary, T_p and T_{ko} provide two independent variables for studying the putative association between temperature and those gene arrangements that show latitudinal clines and long-term trends correlated with current climate change.

CHROMOSOMAL INVERSION POLYMORPHISM

Gene arrangement frequencies

The frequencies of chromosomal gene arrangements of *D. subobscura* are shown in Appendix 1, together with summary statistics for each trait variable analyzed. The gene arrangements observed were generally those already present in prior samples made in 1985–1986 from Portuguese populations near Adraga (Brehm and Krimbas 1987), but warm-adapted gene arrangements tended to increase in frequency in accordance with the trends detected by Solé et al. (2002) in southwestern Europe. When grouped as cold- or warm-adapted, the overall frequencies (no sex difference was detected in any case; results not shown) of cold-adapted gene arrangements are 0.176 for chromosome A, 0.229 for chromosome J, 0.014 for chromosome U, 0.320 for chromosome E, and 0.116 for chromosome O. All chromosomes can therefore be considered polymorphic by the 0.99 criterion.

Association between thermal preference in the laboratory and gene arrangements

Figure 4 presents the results for the association analyses when grouping the segregating gene arrangements in the dataset into cold- or warm-adapted categories. Two-way ANCOVAs performed for each chromosome, with sex and gene arrangement as categorical predictors (after correcting for batch effects) and hour of the thermal preference assay as a covariate indicated that females had a higher T_p than males (see above), but no significant sex × gene arrangement interaction was detected in any case (results not shown). The (parametric) results for the gene arrangement main effect were as follows. (1) Chromosome A: $F = 12.29$, df = 1, 1099, $P < 0.001$. (2) Chromosome J: $F = 0.04$, df = 1, 1103, $P = 0.844$. (3) Chromosome U: $F = 1.66$, df = 1, 1089, $P = 0.198$. (4) Chromosome E: $F = 0.49$, df = 1, 902, $P = 0.485$. (5) Chromosome O: $F = 3.68$, df = 1, 738, $P = 0.055$. These results were checked with permutation tests (with 10,000 random permutations after correcting for batch effects, sex, and hour of the thermal preference assay) and the qualitative conclusions remain (chromosome A: $P = 0.0003$; chromosome J: $P = 0.8213$;

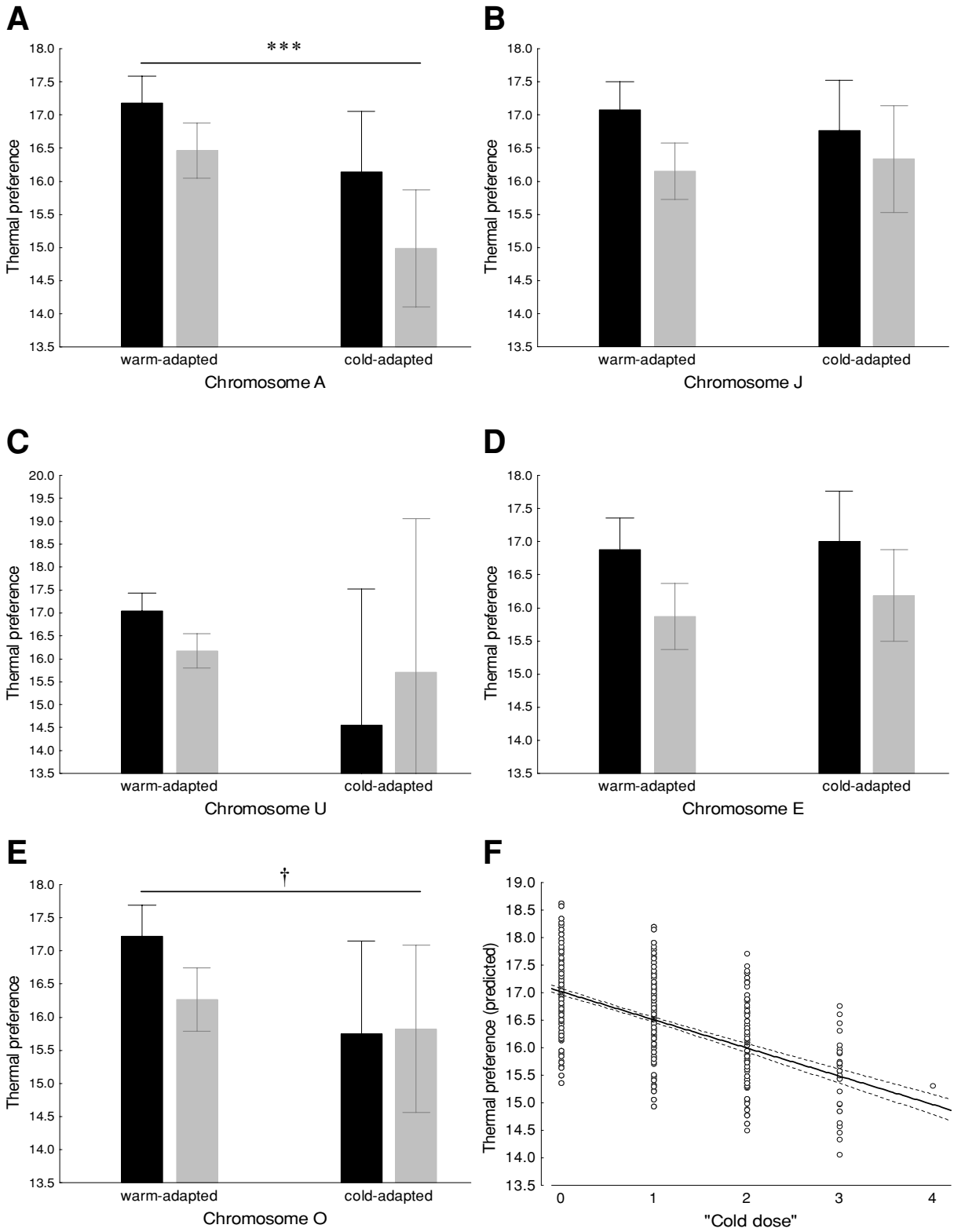

Figure 4. Laboratory thermal preference (T_p in °C with 95% confidence intervals; black bars, females; gray bars, males) in relation to chromosomal gene arrangements grouped as cold- or warm-adapted. Panels (A)–(E) plot the results for each of the five major acrocentric chromosomes (statistical significance for the gene arrangement main effect: $^{\dagger}0.10 > P > 0.05$; $^{***}P < 0.001$; see text for details). Panel (F) plots the relationship (with the 95% confidence region) between the predicted thermal preference and the "cold dose" from a multiple linear regression holding constant the variables sex, batch, and hour of thermal preference assay. Note: in panels (D) and (E) those gene arrangements whose frequencies are uncorrelated with environmental variables were not included in the analysis (see Appendix 1).

chromosome U: $P = 0.1590$; chromosome E: $P = 0.4780$; chromosome O: $P = 0.0737$).

Interestingly, there was a regular trend for individuals carrying cold-adapted gene arrangements to choose colder temperatures, particularly for chromosome A and to a lesser extent for chromosome O (Fig. 4A–E). The global effect of the chromosomes was analyzed by estimating the "cold dose" (Fig. 4F); namely, the number of cold-adapted gene arrangements carried out by a fly, that here can range from 0 to 5 (the expected frequency of flies in class 5 is 2.2×10^{-5} and none was found in the dataset). Note that flies were scored for only one chromosomal set (see Material and Methods) and, hence, there is some noise in the index. Assuming that gene arrangements combine at random both within and between chromosomes (no statistical association between cold-adapted arrangements was found; data not shown), the slope of the regression on the cold dose (estimated from a multiple linear regression holding constant the variables sex, batch, and hour of thermal preference assay) could be taken to be as a rough estimate of how much laboratory thermal preference depends on the number of cold- or warm-adapted chromosomes. The regression coefficient is $\beta_{T_p} = -0.436 \pm 0.173$ ($t = 2.51$, df $= 1114$, $P = 0.012$), which clearly indicates that average T_p decreases with the cold dose and suggests that the expected range of T_p between extreme genotypes, one with 10 cold-adapted gene arrangements (maximum) and the other with none, is $\sim$4°C. In spite of being statistically significant, the amount of variation explained by the cold dose is meager ($R^2 = 7.7 \times 10^{-3}$) and around 1%. (Note however that it represents $\sim$5% of the average intraclass correlation.)

Finally, it might be also interesting to note that there was a consistent negative relationship between T_p—max and the cold dose during the recording time periods (statistically significant for flies' positions after 60 and 70 min in the thermal gradient; see Material and Methods), which suggest that flies differentially avoided high temperatures according to their genetic constitution.

Association between knockout temperatures and gene arrangements

Figure 5 shows the results of the relationship between T_{ko} and the chromosome constitution when grouping the segregating gene arrangements in the dataset into cold- or warm-adapted categories. Two-way ANOVAs with sex and gene arrangement as categorical predictors (after correcting for batch effects and water bath) did not detect a significant sex × gene arrangement interaction in any case (results not shown). The results for the gene arrangement main effect were as follows. (1) Chromosome A: $F = 0.20$, df $=$ 1, 1095, $P = 0.653$. (2) Chromosome J: $F = 0.82$, df $= 1$, 1099, $P = 0.367$. (3) Chromosome U: $F = 0.14$, df $= 1$, 1086, $P = 0.711$. (4) Chromosome E: $F = 12.93$, df $= 1$, 898, $P <$ 0.001. (5) Chromosome O: $F = 0.24$, df $= 1$, 736, $P = 0.625$. These results were also checked with permutation tests and the same qualitative conclusions were obtained (chromosome A: $P =$ 0.6756; chromosome J: $P = 0.3688$; chromosome U: $P = 0.6646$; chromosome E: $P = 0.0004$; chromosome O: $P = 0.5873$).

There was a regular trend for individuals carrying cold-adapted gene arrangements to have a lower heat stress tolerance (significantly so for chromosome E, Fig. 5A–E). We have also analyzed the global effect of the chromosomes on T_{ko} using the cold dose (Fig. 5F), and the slope of the regression (estimated from a multiple linear regression holding constant the variables sex, batch, and water-bath) is $\beta_{T_{ko}} = -0.131 \pm 0.053$ ($t = 2.47$, df $=$ 1110, $P = 0.013$). As for T_p, the amount of variation explained by the cold dose on T_{ko} was also low ($R^2 = 7.3 \times 10^{-3}$ or around 1%).

Discussion

EVOLUTION OF THERMAL PREFERENCES

Although a variety of factors can influence T_p of *Drosophila* flies, stable differences among lines can persist after various generations under laboratory rearing conditions thus indicating genetic differences in T_p (reviewed in Dillon et al. 2009). Assuming that the intraclass correlation reflects the upper bound of T_p heritability, our figures seem to be within the expected range in view of the low mean heritability for behavioral traits in *Drosophila* (mean ± SD: $h^2 = 0.18 \pm 0.26$; see Roff and Mousseau 1987), although Yamamoto (1994b) reported a high "heritability" (0.81) for thermal preference in *D. immigrans*. This figure is however upwardly biased because parental genotypes in Yamamoto's diallel analysis were not taken at random from a base population but were selected on the basis of their extreme thermal preferences.

When replicated populations of the temperate (i.e., cold-tolerant; see David et al. 2003) species *D. subobscura* were allowed to evolve at a constant temperature of 13°C, 18°C, or 22°C, we assumed that the thermal selection regimes spanned much of its tolerable range, and that 18°C was the approximate thermal optimum (Santos et al. 2004, 2005). The present results prove that our characterization was not farfetched because average T_p is around that value (Fig. 2) and certainly lower than the recent estimate of 23.7°C (Huey and Pascual 2009). Although thermal responses can vary between populations (e.g., Yamamoto 1994a) and are affected by various factors (feeding, starvation, reproductive state; see Dillon et al. 2009), it seems to us that the $\sim$7°C−8°C difference between T_p estimates is too large and warrants some explanation (note that our highest T_{set} bound at 20.8°C is below their lower bound at 21.2°C). The study by Davis et al. (1998b) might shed some light here. These authors set up a laboratory system that mimicked a 5°C step latitudinal thermal cline from 10°C to 25°C, and studied dispersal in one- and three-species clines using three *Drosophila* species: *D. melanogaster*, *D. simulans*, and *D. subobscura*. The temperature optimum for *D. subobscura*,

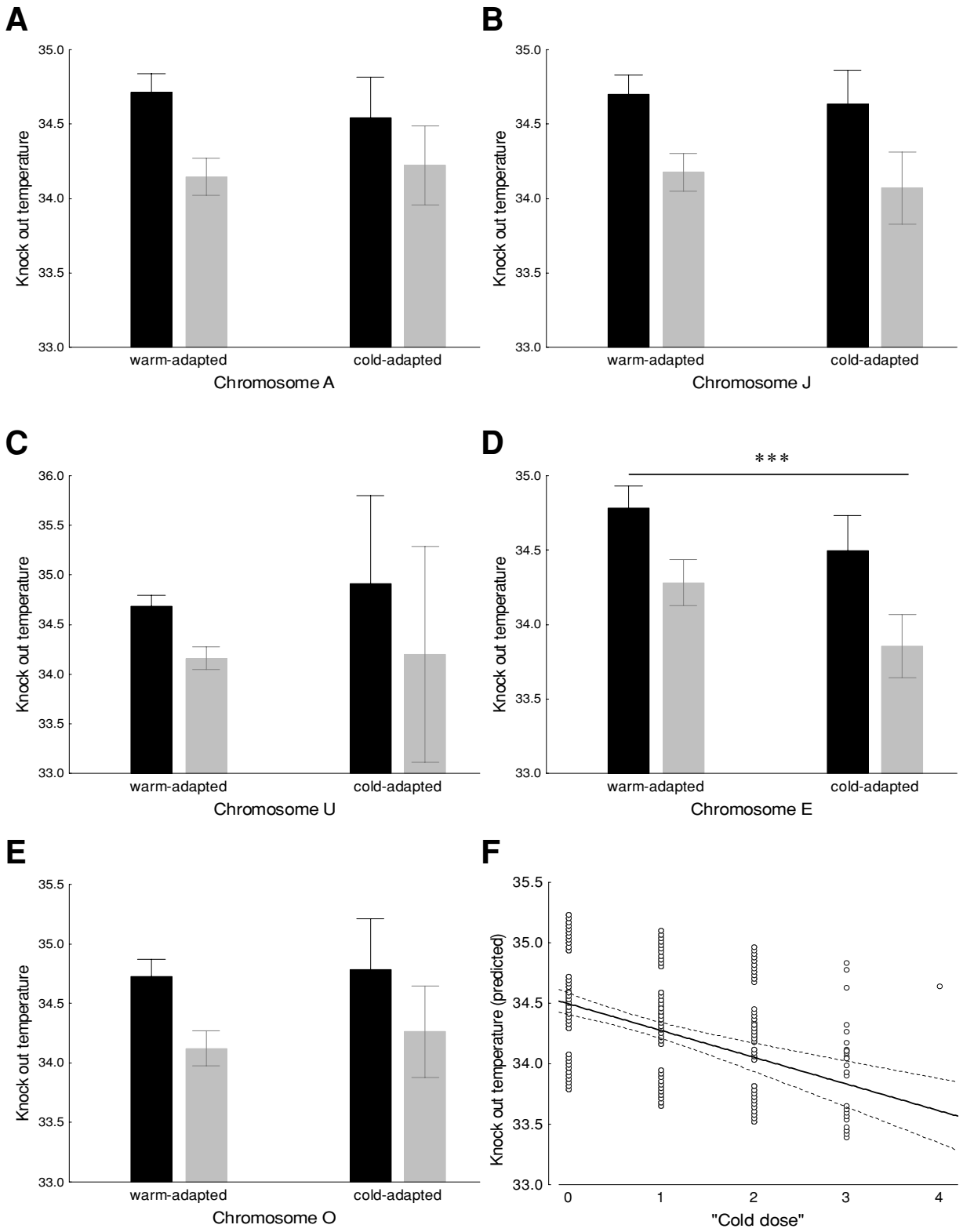

Figure 5. Knockout temperature (T_{ko} in °C with 95% confidence intervals; black bars, females; gray bars, males) in relation to chromosomal gene arrangements grouped as cold- or warm-adapted. Panels (A)–(E) plot the results for each of the five major acrocentric chromosomes (statistical significance for the gene arrangement main effect: ***$P < 0.001$; see text for details). Panel (F) plots the relationship (with the 95% confidence region) between the predicted knockout temperature and the "cold dose" from a multiple linear regression holding constant the variables sex, batch, and water-bath. Note: in panels (D) and (E) those gene arrangements whose frequencies are uncorrelated with environmental variables were not included in the analysis (see Appendix 1).

calculated from single species populations as the weighted mean of the number of flies at each temperature, was (mean ± SD) 16.2°C ± 0.65°C and almost identical to the pooled average in our dataset (16.6°C). We therefore conclude that our present estimates can be confidently taken to be the actual T_p (or T_{set}) of the species.

The evolution of thermal sensitivity obviously depends on the presence of additive genetic variance, which can be estimated from similarity among relatives and responses to selection (Falconer and Mackay 1996). Assuming that no genetic or ecological constraints exist (e.g., species interactions can shift *D. subobscura* to suboptimum temperatures; Davis et al. 1998a), we can expect that the optimal thermal fitness curve will evolve eventually. Consistent with the association between T_p and some underlying genetic variation (Fig. 4A and E), a significant shift in thermal optima was observed in *D. subobscura* after four years of experimental evolution: flies that evolved at 22°C had the highest mean fitness, whereas flies that evolved at 13°C had the lowest, mainly in the warm environment ("warmer is better"; Santos 2007). Therefore, assuming T_p corresponds closely with temperatures that maximize fitness (but see Martin and Huey 2008), a more focused prediction comes up from our present data regarding the evolution of the chromosomal inversion polymorphism in laboratory cage populations because temperature was the only driving force there, something that is far from clear when dealing with natural populations along a latitudinal gradient (Bradshaw and Holzapfel 2006). Cold-adapted gene arrangements on chromosomes A and O could be expected to increase in frequency in cold-adapted (13°C) populations and/or decrease in frequency in warm-adapted (22°C) populations. Santos et al. (2005, p. 269) noted: "The most obvious feature was a general lack of correspondence between the outcomes from laboratory thermal selection and New World colonizations: the only case where both laboratory and natural trends were coincident is for arrangement O_{3+4+2} (and perhaps O_{st})." Furthermore, a closer look at their Figure 2 shows that in all three replicated lines at the highest temperature the average frequency of A_{st} dropped and was the lowest after two years of thermal evolution. To put it another way, there are indeed some consistent patterns between the population cages outcomes and our results here, which suggest that thermoregulation does affect fitness in *D. subobscura* but also that there is more to latitudinal clines than thermoregulatory behavior.

HEAT STRESS TOLERANCE

In temperate climates, where the environment is more variable and unpredictable than in tropical or marine environments, tolerance to thermal limits may be of critical importance for the survival and dispersal of species. The common observation that tolerance to unusually high or low temperatures varies geographically in *Drosophila* (Coyne et al. 1983; Hoffmann et al. 2003) suggests that genetic variation is available and that thermal sensitivity is likely under selection in natural populations. Adaptive responses to thermal extremes may even evolve in parallel, with tolerance to high temperature increasing and cold resistance decreasing toward the tropics (Hoffmann et al. 2002; but see Huey and Kingsolver 1993). Working with *D. subobscura*, Quintana and Prevosti (1990, 1991) used family selection to alter heat resistance in freshly collected flies from the wild. Response to selection was observed in up and down selected lines for resistance, although resistance levels of the control lines decreased with increasing time in the laboratory probably because of inbreeding. Resistance in the up lines might therefore have not raised much above initial levels prior to selection. They also studied the correlated changes in inversion polymorphism and concluded that there was a quite erratic variation of gene arrangement frequencies in the selected lines. Our present results on T_{ko} temperatures indicate that their findings were not at all unexpected (Fig. 5), although the prediction here is that warm-adapted gene arrangements on chromosome E should probably have increased in frequency in the heat-resistant lines (something that did not happen). What could be the reason for chromosome E to have a large effect on heat resistance, as suggested from our data? A tentative answer comes from the fact that chromosome E in *D. subobscura* is homologous to arm 2R in *D. melanogaster*, where the single copy *Drosophila hsf* gene encoding for the heat shock transcription factor (HSF) is located (Clos et al. 1990). Papaceit et al. (2006) have recently compared the chromosomal organization of arm 2R in *D. melanogaster* and chromosome E in *D. subobscura*, and confirmed that all probes that gave a positive result in cross-hybridizations mapped on these homologous chromosomal elements (see also Laayouni et al. 2007). The HSF factor is apparently dispensable for general cell growth or viability under normal conditions (Jedlicka et al. 1997), but is essential for the regulatory response to elevated temperatures by rapidly inducing heat shock protein expression that is known to covary with thermal habitat in a wide variety of species (Krebs and Feder 1997; Feder and Hofmann 1999; Lerman and Feder 2001; Sørensen et al. 2001). It remains to be seen if the response here is consistent across populations, and to what extent HSF is involved in the genetic variation to heat knockout resistance in *D. subobscura*.

CHROMOSOMAL INVERSION CLINES

Understanding the evolutionary responses of organisms to spatial and temporal changes in temperature obviously requires a multidimensional approach. We have captured two components of this multivariate space linked to chromosomal inversion polymorphisms correlated with thermal adaptation in natural populations. The nearly orthogonality between T_p and T_{ko} suggests that both traits are genetically uncorrelated, which agrees with the observation that different independently segregating chromosomes were

mainly responsible for the associations with thermal preference and knockout temperature in the laboratory. How can we relate our findings with the latitudinal variation in gene arrangement frequencies? Was fitness-dependent dispersal important in shaping the quickly evolved New World latitudinal clines as suggested by Prevosti et al. (1985)? In a hypothetical scenario, we can assume that *D. subobscura* colonizers dispersed first at random with respect to segregating gene arrangements and selection favored different thermal fitness optima afterwards. Inversion clines could then be established by stabilizing locally adapted alleles against gene exchange with migrants from other populations, particularly if there is positive synergism among those alleles (Kirkpatrick and Barton 2006; Santos 2009). In line with the results from the laboratory population cages (see above), warm-adapted gene arrangements on chromosomes A and O could be expected to decrease in frequency at higher latitudes at a faster rate than warm-adapted gene arrangements on other chromosomes because these chromosomes were correlated with T_p differences, and T_p is assumed to correspond closely with temperatures that maximize fitness. It turns out to be the case that the fastest-evolving clines following the arrival of *D. subobscura* in Chile were those on chromosomes E, U, and O, although the only one that consistently remained (i.e., was statistically significant in all surveys) after two decades since the South American invasion was for chromosome E (the same trend was found in North American flies; see Balanyà et al. 2003). It is therefore tempting to conclude that selection on tolerance to thermal extremes is more important in the evolution and dynamics of clinal patterns than the relatively "minor" adjustments from behavioral thermoregulation, particularly if *Drosophila* thermoregulatory behavior does in fact decouple mean T_b and mean environmental temperature to somewhat mitigate the impact of spatial variation in thermal environments.

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Supporting Information

The following supporting information is available for this article:

Appendix S1. Chromosomal polymorphism

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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APPENDIX 1: Chromosomal polymorphism

Table A1. Chromosomal polymorphism of *Drosophila subobscura* derived from the population at Adraga together with some basic statistics (mean ± SD) for thermal preference (T_p) in the laboratory and knock out temperature (T_{ko}).

	Females			Males		
Gene arrangement [a]	(Count) Freq.	T_p (ºC)	T_{ko} (ºC)	(Count) Freq.	T_p (ºC)	T_{ko} (ºC)
A_{st} (cold)	(87) 0.156	16.0 ± 5.0	34.5 ± 1.3	(94) 0.166	14.8 ± 4.4	34.2 ± 1.4
A_1 (cold)	(10) 0.018	16.4 ± 4.6	34.0 ± 1.4	(7) 0.012	15.3 ± 5.3	33.6 ± 0.8
A_2 (warm)	(460) 0.823	17.3 ± 4.4	34.7 ± 1.3	(464) 0.821	16.5 ± 4.8	34.1 ± 1.6
A_{2+6} (warm)	(2) 0.004	15.3 ± 6.7	34.0 ± 0.0	–	–	–
N	559	557	555	565	557	555
J_{st} (cold)	(136) 0.242	16.8 ± 4.8	34.7 ± 1.5	(122) 0.215	16.2 ± 4.7	34.0 ± 1.5
J_1 (warm)	(425) 0.758	17.2 ± 4.4	34.6 ± 1.2	(445) 0.785	16.2 ± 4.8	34.2 ± 1.6
N	561	559	557	567	559	557
U_{st} (cold)	(9) 0.016	15.0 ± 4.4	34.6 ± 0.9	(7) 0.012	16.0 ± 5.0	34.7 ± 1.0
U_{1+2} (warm)	(190) 0.344	17.8 ± 4.6	34.7 ± 1.3	(209) 0.372	16.5 ± 4.8	34.2 ± 1.6
U_{1+2+8} (warm)	(353) 0.639	16.7 ± 4.4	34.6 ± 1.3	(346) 0.616	16.0 ± 4.8	34.1 ± 1.6
N	552	550	549	562	554	552
E_{st} (cold)	(133) 0.239	17.2 ± 4.2	34.4 ± 1.4	(163) 0.289	16.3 ± 4.5	33.8 ± 1.5
E_8	(5) 0.009	13.6 ± 3.8	35.2 ± 1.1	(2) 0.004	17.4 ± 1.9	36.0 ± 0.0
E_{1+2}	(96) 0.173	17.6 ± 4.8	34.6 ± 1.3	(92) 0.163	17.0 ± 5.3	34.2 ± 1.4
E_{1+2+9} (warm)	(121) 0.218	17.6 ± 4.6	34.8 ± 1.0	(128) 0.227	15.8 ± 4.5	34.4 ± 1.6
$E_{1+2+9+3}$ (warm)	(12) 0.022	18.5 ± 4.7	34.8 ± 1.2	(8) 0.014	15.2 ± 5.3	33.0 ± 1.2
$E_{1+2+9+12}$ (warm)	(189) 0.340	16.4 ± 4.4	34.7 ± 1.4	(171) 0.303	16.0 ± 4.9	34.2 ± 1.7
N	556	554	552	564	556	554

Table A1. (Continued.)

Gene arrangement [a]	Females			Males		
	(Count) Freq.	T_p (°C)	T_{ko} (°C)	(Count) Freq.	T_p (°C)	T_{ko} (°C)
O_{st} (cold)	(38) 0.068	15.4 ± 4.8	34.7 ± 1.1	(50) 0.088	15.5 ± 5.0	34.0 ± 1.5
O_7	(2) 0.004	11.7 ± 0.3	34.0 ± 0.0	(5) 0.009	15.0 ± 6.5	35.0 ± 0.7
O_{3+4} (warm)	(284) 0.508	17.4 ± 4.3	34.7 ± 1.3	(276) 0.488	16.4 ± 4.7	34.2 ± 1.6
O_{3+4+1} (warm)	(31) 0.055	16.3 ± 4.5	34.9 ± 1.3	(35) 0.062	15.7 ± 4.1	33.8 ± 1.5
O_{3+4+2}	(13) 0.023	17.4 ± 5.5	34.3 ± 0.8	(22) 0.039	16.7 ± 5.5	34.0 ± 1.7
O_{3+4+6}	(5) 0.009	16.5 ± 4.0	35.2 ± 1.1	(4) 0.007	14.2 ± 2.5	34.3 ± 1.5
O_{3+4+7}	(157) 0.281	17.1 ± 4.7	34.6 ± 1.4	(153) 0.270	16.2 ± 5.0	34.2 ± 1.6
O_{3+4+8} (warm)	(25) 0.045	17.4 ± 4.3	34.7 ± 1.0	(18) 0.032	16.8 ± 4.0	33.8 ± 1.6
$O_{3+4+23+2}$	(4) 0.007	13.8 ± 2.9	34.8 ± 1.5	(3) 0.005	16.4 ± 4.5	33.0 ± 1.7
N	559	557	555	566	558	556

[a] Gene arrangements are labeled as “cold-adapted” (cold) or “warm-adapted” (warm) based on the study by Menozzi and Krimbas (1992) of the correlation of gene arrangement frequencies and environmental variables in Palearctic populations.