

# Temperature-Related Genetic Changes in Laboratory Populations of *Drosophila subobscura*: Evidence against Simple Climatic-Based Explanations for Latitudinal Clines

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**ABSTRACT:** Parallel latitudinal clines to the long-standing ones in the original Palearctic populations have independently evolved at different rates for chromosomal polymorphism and body size in South and North American populations of *Drosophila subobscura* since colonization around 25 years ago. This strongly suggests that (micro) evolutionary changes are largely predictable, but the underlying mechanisms are unknown. The putative role of temperature per se was investigated by using three sets of populations at each of three temperatures (13°, 18°, and 22°C) spanning much of the tolerable range for this species. We found a lower chromosomal diversity at the warmest temperature; a quick and consistent shift in gene arrangement frequencies in response to temperature; an evolutionary decrease in wing size, mediated by both cell area and cell number,

at 18°C; no relationship between wing size and those inversions involved in latitudinal clines; and a shortening of the basal length of longitudinal vein IV relative to its total length with increasing standard dose. The trends for chromosomal polymorphism and body size were generally inconsistent from simple climatic-based explanations of worldwide latitudinal patterns. The findings are discussed in the light of available information on *D. subobscura* and results from earlier thermal selection experiments with various *Drosophila* species.

**Keywords:** chromosome polymorphism, clinal variation, *Drosophila subobscura*, thermal evolution, wing size, wing shape.

Latitudinal and altitudinal clines are prominent indicators of natural selection at work over large geographic scales. Some classical examples from *Drosophila* species are gradients in chromosomal inversion frequencies (e.g., Dubinin and Tiniakov 1946; Stalker 1976; Mettler et al. 1977; Levitan 1978; Knibb et al. 1981; Menozzi and Krimbas 1992; van't Land et al. 2000; Schaeffer et al. 2003; see Krimbas and Powel 1992 for review) and geographical clines in body size with genetically larger individuals derived from higher latitudes (Stalker and Carson 1947; Prevosti 1955; Misra and Reeve 1964; David et al. 1977; Coyne and Beecham 1987; Pegueroles et al. 1995; James et al. 1997; van't Land et al. 1999; Huey et al. 2000). The most convincing evidence that such clines are adaptive probably comes from the temperate (i.e., cold tolerant) species *Drosophila subobscura*.

Formerly restricted to the Palearctic region, these flies were discovered in South America at the locality of Puerto Montt (Chile) in February 1978 (Brncic and Budnik 1980) and explosively colonized a broad latitudinal range (Brncic et al. 1981; Prevosti et al. 1985). Also remarkable was the discovery in 1982 of *D. subobscura* in North America near Port Townsend, Washington (Beckenbach and Prevosti 1986), because those two colonization events could now

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be considered a large-scale natural experiment with two replicates (i.e., “a grand experiment in evolution”; Ayala et al. 1989). The regular tracking of the inversion polymorphism and body dimensions ever since the colonization of the Americas by *D. subobscura* has resulted in a number of evolutionarily important findings (summarized in Gilchrist et al. 2001, 2004; Serra 2002; Balanyà et al. 2003). First, almost all inversions that occur in New World populations have shifted in frequency as a response to environmental gradients, and latitudinal clines were soon detected. Second, those inversions show the same correlation sign of frequency with latitude as in the original Old World populations. Third, inversion clines have not continued to converge on the native Old World baseline. Fourth, after approximately 20 years from introducing the species into South and North America, geographical clines have also evolved for wing length that are morphologically equivalent to the original cline in the Old World; however, fifth and finally, the underlying details by which the body size clines were achieved—either the cellular basis of different size clines (Calboli et al. 2003a) or the particular wing segment causing the cline (Huey et al. 2000)—seem to be contingent (but see Santos et al. 2004). Therefore, the conclusion that the inversion polymorphism and body size of *D. subobscura* are selectively responding to worldwide parallel environmental conditions is quite compelling.

The repeatability of body size clines across continents and *Drosophila* species, together with laboratory experiments on thermal evolution where adaptation to lower temperature consistently resulted in increased body size (Anderson 1966; Powell 1974; Cavicchi et al. 1989; Partridge et al. 1994a), has led most researches to conclude that geographical clines in *Drosophila* body size are indeed the result of adaptation to different temperatures (albeit a thorough understanding of temperature-dependent body size variation in ectotherms is still lacking). The snag is, however, that unintended selection due to failures in controlling conditions in life cycle stages that are not directly targeted by the selection procedures can spoil the interpretation of results (see Rose et al. 1996). In the aforementioned thermal selection experiments, the most commonly neglected variable was larval culture density across temperatures. For instance, in *Drosophila melanogaster*, it is well known that populations stabilize at lower densities when maintained at lower temperatures, and, hence, crowding conditions differ between thermal selection stocks (L. Partridge, personal communication, 1998). Spurious temperature-related adjustments of adult body size may then result because physiological adaptations to temperature were not independent of adaptations to different levels of larval crowding, which, in turn, involve changes in larval behavior and physiology that may impinge on

other phases of the life cycle (see, e.g., Joshi and Mueller 1996; Santos 1996; Santos et al. 1997; Houle and Rowe 2003). In summary, most laboratory thermal selection experiments carried out so far have the drawback that different levels of harmful larval waste products (Borash et al. 1998, 2000) and food shortage due to various crowding conditions co-varied with selection pressures in shaping larval resource processing at various temperatures (Bochdanovits and de Jong 2003a, 2003b).

With the aim of studying the short- and long-term outcomes of thermal selection on the chromosomal inversion polymorphism and wing size and shape of *D. subobscura*, we have developed a set of three replicated populations kept at three experimental temperatures under controlled larval densities (see Santos et al. 2004): cold (13°C), optimum (18°C), and warm (22°C). These populations have been sampled twice: after 1 and 2 years since foundation when they had already diverged for 27 and 51 generations at most, respectively. The results certainly show that the chromosomal inversion polymorphism has quickly and consistently responded to thermal regime, but the trends were generally not in harmony with predictions based on latitudinal clines or continent-wide shifts putatively caused by global warming (e.g., Solé et al. 2002). Contrary to what had been observed in the first sample, temperature-related genetic differences in wing size were detected after 2 years, but warm-adapted populations were not smaller than their cold-adapted counterparts and had about the same wing cell area. Finally, we provide convincing evidence that New World latitudinal clines for polymorphic inversions and body size in *D. subobscura* are clearly uncoupled. We discuss the results in the light of available information on the inversion polymorphism of *D. subobscura* and also challenge the idea that *Drosophila* body size is the target of thermal selection.

## Material and Methods

### *Experimental Populations and Sampling Protocol*

All nine populations used here were initiated from an ancestral population of *Drosophila subobscura* derived from a large outbred stock collected in November 1999 at the estimated Chilean epicenter of the original New World invasion (Puerto Montt, Chile, 41°28'S; see Brncic and Budnik 1980). From that ancestral population, three sets (13°, 18°, and 22°C) of three replicate populations each (R1, R2, and R3) were set up in May 2001. These thermal selection stocks are continuously kept on a discrete generation, controlled larval crowded regime on a 12L : 12D period. Prior to initiating a new generation, eclosed adults from the bottles are dumped into a Plexiglas cage (27 cm × 21 cm × 16 cm) and supplied with liberal amounts

of food before egg collections. The number of breeding adults per population is typically well over 1,500 flies. Complete details of the derivation and maintenance of these populations have previously been described (Santos et al. 2004).

Simultaneously to the establishment of the thermal selection stocks, a large sample of eggs were collected to estimate the frequencies of the chromosomal arrangements in the founding population. Samples from all nine populations were next taken after 1 (May 2002: nine generations at 13°C, 12 at 18°C, and 15 at 22°C) and 2 years (April 2003: 16 generations at 13°C, 22 at 18°C, and 29 at 22°C) of thermal selection (see Santos et al. 2004 for experimental details). In order to control for the possibility of nongenetic parental effects on offspring size (Crill et al. 1996), the parents of sampled flies had also been reared at the same temperature of 18°C. Experimental flies were separated by sex; females were stored in Eppendorf tubes with a 3:1 mixture of alcohol and glycerol at 4°C, and males (125–150 males per population randomly chosen from the 12 replicated bottles) were individually crossed in vials (2 cm × 8 cm containing 6 mL of food) to three to four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies (see below). After approximately 9 days, the males were removed from the vials and individually fixed in a 3:1 mixture of alcohol and glycerol at 4°C. All fly handling was done at room temperature using CO<sub>2</sub> anesthesia on flies not less than 6 h after eclosion.

#### Chromosomal Inversions

The karyotype of *D. subobscura* consists of five acrocentric chromosomes and a dot chromosome. Following Mainx et al. (1953), the large chromosomes in this species are traditionally named as A (=X, the sex chromosome), J (=chromosomal element D of Mueller/Sturtevant/Novitski and homologous to arm 3L in *Drosophila melanogaster*; see Powell 1997, p. 307), U (=chromosomal element B and homologous to arm 2L), E (=chromosomal element C and homologous to arm 2R), and O (=chromosomal element E and homologous to arm 3R). In colonizing populations of the New World, a total of 18 chromosomal arrangements (see Balanyà et al. 2003) have been found out of approximately 92 (produced from 66 inversions) recorded in the Palearctic region (Krimbas 1992, 1993; Menozzi and Krimbas 1992). All those 18 arrangements were present in the sample from Puerto Montt used to derive the thermal selection stocks: namely, A<sub>st</sub>, A<sub>2</sub>, J<sub>st</sub>, J<sub>1</sub>, U<sub>st</sub>, U<sub>1+2</sub>, U<sub>1+2+8</sub>, E<sub>st</sub>, E<sub>1+2</sub>, E<sub>1+2+9</sub>, E<sub>1+2+9+3</sub>, E<sub>1+2+9+12</sub>, O<sub>st</sub>, O<sub>3+4</sub>, O<sub>3+4+2</sub>, O<sub>3+4+7</sub>, O<sub>3+4+8</sub>, and O<sub>5</sub> (for complete details of the naturally occurring inversions in *D. subobscura*, see Krimbas and Loukas 1980; Krimbas

1993). In addition, arrangement O<sub>7</sub> was also present at Puerto Montt but at a very low frequency (0.7%; see table A1 in the online edition of the *American Naturalist*). This arrangement, which is also found periodically in the Old World, is probably the result of a recombination event in the O<sub>3+4+7</sub>/O<sub>st</sub> heterokaryotype.

In *D. subobscura*, it is difficult to recognize the gene arrangements in all possible homozygous/heterozygous combinations. Therefore, males were individually crossed to three or four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies. This strain is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu*; Koske and Maynard Smith 1954), and its genetic background is highly homogeneous and fixed for the standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O<sub>3+4</sub> (Lankinen and Pinsker 1977). Whenever feasible, one F<sub>1</sub> female third-instar larva derived from each cross with the homozygous *ch-cu* stock was examined for its inversion loops in polytene chromosomes to determine the gene arrangements of one set of the chromosomes from the wild-type male. Sample sizes were always larger than 100 gametes per population (table A1).

#### Wing Measurements

Wing size and shape of both sexes from each experimental population were measured as previously described (Santos et al. 2004). Briefly, wings were removed from each fly and fixed in DPX under coverslips on microscope slides. A total of ~100 females per population selected haphazardly from the 12 replicated bottles (see above), as well as the majority of males crossed to the *ch-cu* marker strain for chromosomal study, were used for measurements. Bit-map images of the wings were captured with a Sony CCD-Iris video camera connected to a PC with MGI VideoWave software and mounted on a Zeiss Axioskop compound microscope, using a 2.5× objective. The images were stored on a Dell Workstation PWS350 computer and then used to record the *x* and *y* coordinates of 13 morphological landmarks (fig. A1 in the online edition of the *American Naturalist*), using the Scion Image for Windows software (<http://www.scioncorp.com>). All the data used here are from the left wings (which happen to be slightly bigger than the right wings; Iriarte et al. 2003), and all wings have been measured by M. Santos. Using the original landmark coordinates, wing size was estimated in two different ways: as centroid size (CS) and as wing length (WL). The CS is defined as the square root of the sum of squared distances of a set of landmarks from their centroid or, equivalently, the square root of the sum of the variances

of the landmarks about that centroid in  $x$  and  $y$  directions (Slice et al. 1996). Following Robertson and Reeve (1952) and Prevosti (1955), WL was estimated as the combined lengths of the basal (labeled as L1) and distal (labeled as L2) segments of longitudinal vein IV (fig. A1). Wing shape was also estimated as the ratio of the basal length of longitudinal vein IV to the total wing length (i.e., L1/WL). The reason is that these linear measurements have been previously used to study size and shape clines in *D. subobscura* (e.g., Pegueroles et al. 1995; Huey et al. 2000; Gilchrist et al. 2001, 2004).

To estimate the cellular components of body size differentiation, a random sample of the slides including 50 females and 50 males per population already used for wing measurements was sent to Bologna. An image of the left wing was taken by V. Trotta at  $40 \times 10$  magnification, and a sampling square of  $11.55 \times 10^{-3} \text{ mm}^2$  was selected in the area of the wing proximal to the posterior crossvein. Trichome counting followed a standard protocol; namely, the sampling area was visually inspected and the trichomes whose roots were within the selected square were marked with a black dot. Further manipulation provided a final image showing only the dots, which were counted using the ImageJ 1.31 software (<http://rsb.info.nih.gov/ij/>). Cell area was then estimated as  $11.55 \times 10^{-3} \text{ mm}^2/\text{dot number}$ . Because cell area is variable across the wing blade, a total cell number index was estimated as  $(L1 + L2)^2 (\text{mm}^2)/\text{cell area} (\text{mm}^2)$ .

### Statistical Methods

**Chromosomal Diversity.** Chromosomal diversity was computed in three ways: as the number of segregating gene arrangements  $n_i$ ; as the expected heterozygosity  $H_E = 1 - \sum_{i=1}^k p_i^2$ , where  $p_i$  is the relative frequency of gene arrangement  $i$ ; and as  $1 - \text{IFR} = \sum_{i,j=1, i \neq j}^k p_i p_j (1 - l_{ij})$ , where IFR is the index of free recombination (Carson 1955) and  $l_{ij}$  is the proportion of euchromatin that freely recombines between gene arrangements  $i, j$  on the same chromosome. This index, which is claimed to be less biased than the mean number of inversions for which an individual is heterozygous (Krimbas and Loukas 1980; Krimbas 1993; Rodríguez-Trelles and Rodríguez 1998), is basically a measure of heterozygosity weighted by the relative length of the inversion, and a drop in  $1 - \text{IFR}$  means that the population is becoming less heterogeneous for chromosomal arrangements. To compute the index, each heterokaryotype class was considered in turn, and the amount of euchromatin that is involved in inversion loops was obtained from the reference tables that measure the relative lengths of the inverted regions (Krimbas and Loukas 1979). For the total set of chromosomes,  $1 - \text{IFR}$  was estimated as the weighted averages also taking into account

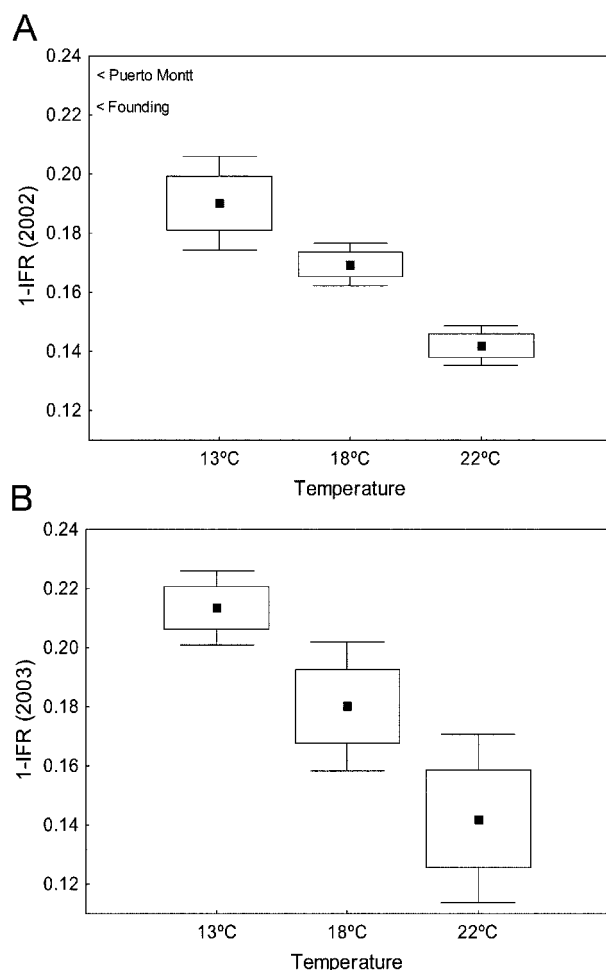
the relative contents of total euchromatin in each chromosome element (see Wasserman 1982).

**Inversion Polymorphism.** The frequencies of chromosomal gene arrangements in the original natural population at Puerto Montt, in the initial founding population, and in the thermal selection stocks after 1 and 2 years of divergence are given in table A1. Data for the first year (May 2002) have been previously published but with the only purpose of relating male wing shape index L1/WL to the standard dose (i.e., the number of standard gene arrangements carried out by a male; see Santos et al. 2004) and are also included in table A1 for completeness.

The unit of analysis here is the population, and the three replicated populations (R1, R2, and R3) of each thermal selection stock were treated as a random factor nested within experimental temperature ( $13^\circ$ ,  $18^\circ$ , and  $22^\circ\text{C}$ ), which was a fixed effect. This nested design somewhat precludes the use of three-way log-linear analyses (including experimental temperature, replicate, and gene arrangement as the main effects) to investigate variation in chromosomal arrangement frequencies because the replicated populations provide the appropriate error term to test for temperature effects (see Sokal and Rohlf 1995).

Since the various gene arrangements can be divided in two groups based on the correlation of gene arrangement frequencies and latitude, with  $A_{st}$ ,  $J_{st}$ ,  $U_{st}$ ,  $E_{st}$ , and  $O_{st}$  included in the “cold-adapted” group (see Menozzi and Krimbas 1992; Balanyà et al. 2003), we can test the prediction that standard chromosomes are expected to decrease in frequency from  $13^\circ\text{C}$  to  $22^\circ\text{C}$  in the thermal selection stocks. The indicator variable  $x_{ij}$ , defined as  $x_{ij} = 1$  if chromosome arrangement  $j$  in male  $i$  was standard and  $x_{ij} = 0$  otherwise, was used in the analyses. Thus, the amount of variation for temperature effects in these analyses is the same as that obtained when using the standard gene arrangement frequency for a given chromosome weighted by the corresponding sample sizes, but we can further test for variation between replicated populations. In addition, the two degrees of freedom for temperature were partitioned into linear (regression) and nonlinear (deviation) effects. Permutation tests were used to assess whether the arrangement frequencies show significant variation in relation to temperature. For the two-level nested ANOVA model, randomization is a two-stage process: random permutations among replicate within temperature for the replicate in temperature  $F$  statistics, and random permutations among replicate and selection temperature for the among selection temperature  $F$  statistics. Each test used 10,000 random permutations of the observations.

Furthermore, year was introduced as a fixed effect in the previous two-level nested ANOVA to evaluate whether the slopes of inversion frequencies on experimental tem-



**Figure 1:** Boxplots showing the average chromosomal diversity (boxes:  $\pm$  SE; whiskers:  $\pm$  SD) for the total set of chromosomes of *Drosophila subobscura* in the thermal selection stocks. Chromosomal diversity was estimated here as  $1 - \text{IFR}$ , where IFR is the index of free recombination (the relative contents of total euchromatin in each chromosome element were taken into account). The  $1 - \text{IFR}$  values from the original population at Puerto Montt and from the founding population are also pointed in A.

perature were similar in the two samples. The interaction between temperature and year should be significant whenever slopes had changed in steepness. In the two-way ANOVA, randomization is a three-stage process (Good 1994; Edgington 1995), and the temperature  $\times$  year interaction effect was tested from random permutations across temperatures and years after subtracting the deviations due to both main factors and adding the grand mean (see Sokal and Rohlf 1995). As before, each test used 10,000 random permutations.

**Wing Variables.** The wing traits measured (WL, CS, cell

size, and cell number index  $[\text{L1} + \text{L2}]^2/\text{cell area}$ ) were analyzed separately (but see below). For each trait we used a standard linear model with replicated populations nested within experimental temperature, which was considered as a fixed effect. In addition, we tested the suggestion that *D. subobscura* wing size (WL) increases with the standard gene arrangement dose (Prevosti 1967; Orengo and Prevosti 2002), as well as our previous finding that polymorphic inversions have a biometric effect on the wing shape index  $\text{L1}/\text{WL}$  (Santos et al. 2004).

The computer programs used for statistical data analyses were MATLAB (MathWorks 2002) together with the collection of tools supplied by the Statistics Toolbox (MathWorks 2000). Results based on parametric ANOVAs were checked with the statistical software packages STATISTICA V.6 (StatSoft 2003) and SPSS (SPSS 2001).

## Results

### Chromosomal Diversity

A potentially important problem in our thermal selection stocks could be the loss of some or many of the naturally segregating polymorphic inversions in *Drosophila subobscura* in the relatively constant laboratory environment—as seems to happen with *Drosophila melanogaster* (e.g., Inoue 1979; Inoue et al. 1984)—for reasons unrelated to temperature adaptation. We have, therefore, first estimated the chromosomal diversity in the original population at Puerto Montt, in the founding population, and in the thermal stocks after 1 and 2 years since their establishment.

As formerly indicated, all  $n_1 = 18$  arrangements present in the New World were found at Puerto Montt, as well as the recombination-derived  $O_7$ , which was also sporadically uncovered in the experimental populations (see table A1). Gene arrangement  $E_{1+2}$ , which had a frequency of 3.7% at Puerto Montt and is not involved in the New World latitudinal clines (Balanyà et al. 2003), was likely lost in the founding population because it has not reappeared in any of the 2,295 slides scrutinized so far for chromosome E. All other gene arrangements (i.e.,  $n_1 = 17$ ) are still segregating in the thermal stocks at various frequencies. Even the allelic lethal-carrying  $O_5$  (Mestres and Serra 1995; Mestres et al. 2001; Iriarte et al. 2003), initially at a frequency of 5%, was recorded five times in 2002 (0.5%) and four times in 2003 (0.4%; table A1).

Figure 1 plots the chromosomal diversities (estimated as  $1 - \text{IFR}$ ) from the time of sampling at Puerto Montt in November 1999. Average chromosomal diversity at 13°C (0.190 in May 2002; 0.214 in April 2003) has remained close to the initial level in the founding population (0.225). However, it is quite obvious that there was a decreasing trend of chromosomal diversity with increasing tem-

perature. The Scheirer-Ray-Hare two-way nonparametric ANOVA (Sokal and Rohlf 1995, pp. 445–447) rendered a statistically significant temperature effect ( $H = 6.37$ ,  $P = .041$ ), but not year ( $H = 0.56$ ,  $P > .05$ ) or temperature  $\times$  year interaction ( $H = 0.03$ ,  $P > .05$ ) effects.

#### Variation in Chromosome Arrangement Frequencies

Average frequencies for the standard chromosome arrangements in the thermal stocks are plotted in figure 2. Temperature effects were statistically significant for  $A_{st}$  and, most remarkably,  $E_{st}$  (table A2 in the online edition of the *American Naturalist*). Arrangement  $A_{st}$  showed a decline in frequency with increasing temperature, mainly due to the sharp drop at 22°C after 29 generations (fig. 2). On the other hand, the initial frequency of  $E_{st}$  dropped from 61.2% to 49.4% after nine generations at 13°C ( $\Delta p = -0.0130$  per generation) and rose up to 82.6% after 15 generations at 22°C ( $\Delta p = 0.0143$  per generation). The slope of the linear regression from the April 2003 sample was about the same as that in May 2002 (table A2); however, the baseline frequency (13°C) of  $E_{st}$  has further decreased at a similar rate from 49.4% in May 2002 to 40.2% in April 2003 ( $\Delta p = -0.0132$  per generation). Conversely,  $E_{st}$  has remained at about the same frequency at 22°C (82.6% in May 2002 vs. 79.8% in April 2003). After an initial increase to 70.9% in May 2002, the frequency of  $E_{st}$  at 18°C had dropped in April 2003 (60.1%) to approximately the same level as that estimated in the founding population. All those figures suggest that the temperature-related shifts in  $E_{st}$  frequency mainly occur within the first ~15 generations. Thus, recall that in May 2002 the stocks at 22°C had already diverged for 15 generations since foundation, and in April 2003, those at 13°C had diverged for 16 generations. The per generation shift in frequency has been about the same but in reverse direction at the two extreme temperatures, so that their midpoint after 15–16 generations was exactly equal to the frequency of  $E_{st}$  in the founding population. The remaining standard gene arrangements for autosomes J, U, and O showed a nearly flat relationship with temperature, with the only exception perhaps being arrangement  $O_{st}$ , whose frequency has also dropped below 5% at 22°C (fig. 2).

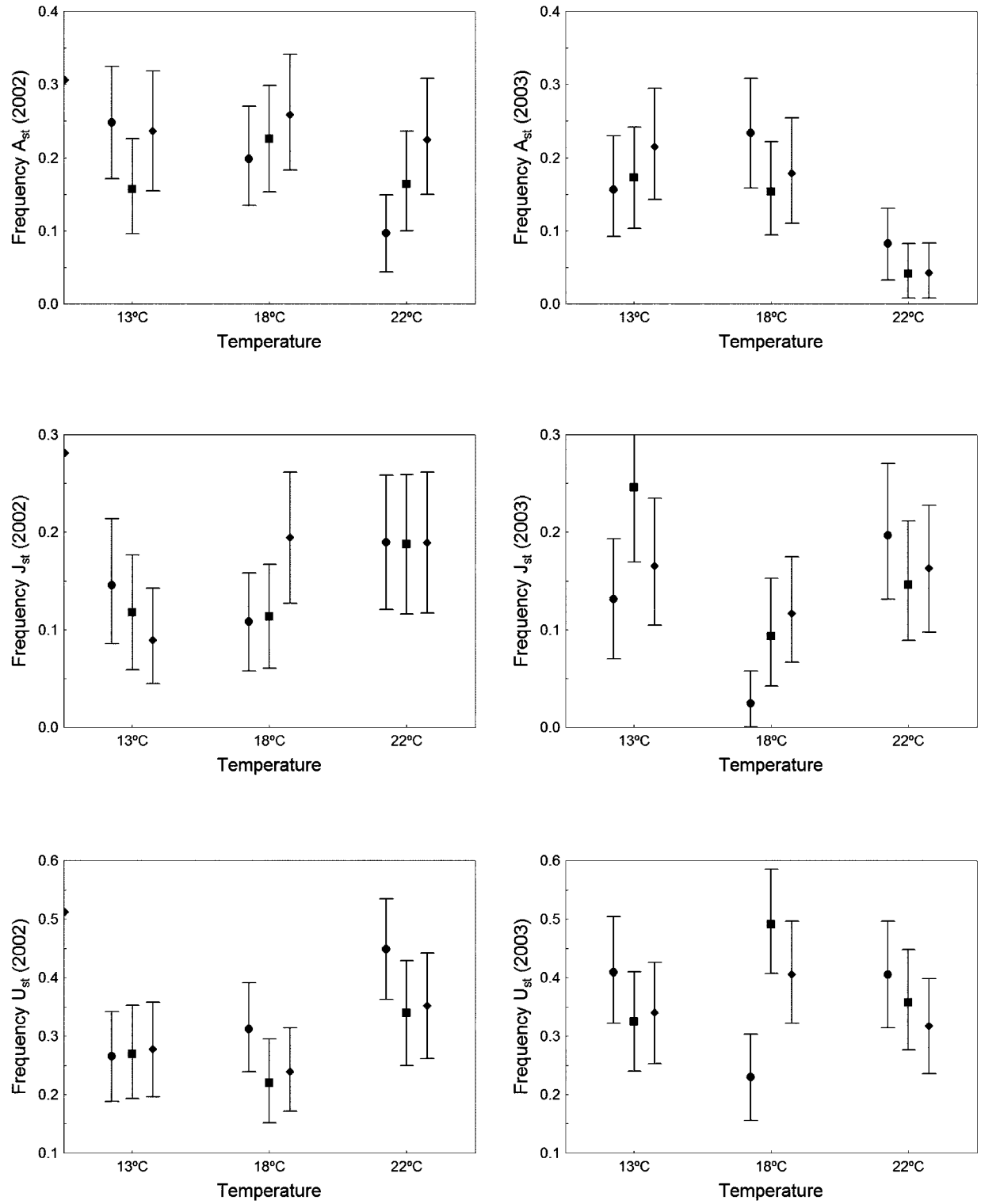
Obviously, inversions  $A_2$  and  $J_1$  show patterns that are just the opposite of those observed for  $A_{st}$  and  $J_{st}$ . For the remaining three chromosomes, the situation is more complex. Aside from “random” fluctuations among replicated populations and/or sampling years, none of the three gene arrangements on chromosome U showed any trend with experimental temperature. Actually, after grouping all laboratory populations, their frequencies in April 2003 (i.e.,  $U_{st} = 36.4\%$ ,  $U_{1+2} = 35.3\%$ , and  $U_{1+2+8} = 28.4\%$ ) were quite similar to those from the natural population at

Puerto Montt ( $U_{st} = 42.2\%$ ,  $U_{1+2} = 34.8\%$ , and  $U_{1+2+8} = 23.0\%$ ). For chromosome E, a flat relationship with temperature was observed for arrangement  $E_{1+2+9}$ , whereas arrangements  $E_{1+2+9+3}$  (May 2002: 18.1% at 13°C, 11.9% at 18°C, and 7.4% at 22°C; April 2003: 26.3% at 13°C, 18.6% at 18°C, and 10.8% at 22°C) and  $E_{1+2+9+12}$  (May 2002: 28.2% at 13°C, 12.9% at 18°C, and 3.2% at 22°C; April 2003: 26.6% at 13°C, 11.9% at 18°C, and 3.5% at 22°C) showed a quite sharp and statistically significant linear decrease in frequency with increasing laboratory temperature. Finally, for chromosome O, arrangement  $O_{3+4}$  dramatically increased in frequency from 5% in the founding population to 21% at 22°C in May 2002. Its frequency in April 2003 was still significantly higher at 22°C (12.8%) than at the lower temperatures, where it remained at approximately the same initial frequency (~6%). Arrangements  $O_{3+4+2}$  and  $O_{3+4+7}$  showed opposite patterns in April 2003: the former significantly increased in frequency, and the latter significantly decreased in frequency at 22°C (table A1). In addition, a conspicuous trend for  $O_{3+4+2}$  has been a general increase in frequency since foundation (from 42.1% to 61.4% in April 2003; table A1). On the other hand, arrangement  $O_{3+4+8}$  did not show any trend with experimental temperature.

#### Thermal Evolution of Wing Traits

**Wing Size.** In contrast with the results after 1 year (Santos et al. 2004), two-level nested ANOVAs of wing size in the samples from April 2003 have already revealed statistically significant differences among thermal selection regimes for both females (WL as  $\log_e [L1 + L2]$ :  $F = 19.64$ ,  $df = 2, 6$ ,  $P = .002$ ; CS [in pixels]:  $F = 23.33$ ,  $df = 2, 6$ ,  $P = .001$ ) and males (WL:  $F = 15.45$ ,  $df = 2, 6$ ,  $P = .004$ ; CS:  $F = 20.09$ ,  $df = 2, 6$ ,  $P = .002$ ). Most striking was the pattern observed between body size and thermal selection regime (fig. 3; only the plots for WL are shown since there was a very high correlation between WL and CS: females  $r = 0.981$ ,  $P < .001$ ; males  $r = 0.980$ ,  $P < .001$ ). Thus, average WL (and CS) was about the same at the two extreme temperatures for both females (Scheffé post hoc tests using the “replicates” mean square as the error term: 13°C [2.7462 mm] vs. 22°C [2.7352],  $P = .646$ ) and males (13°C [2.4717 mm] vs. 22°C [2.4548],  $P = .462$ ) but showed a comparative and statistically significant reduction of ~2% at the optimum temperature of 18°C.

The between-year contrasts for WL should obviously be taken with great caution because of potential differences in the rearing conditions (though exactly the same experimental protocol and incubator shelf at  $18^\circ \pm 0.1^\circ\text{C}$  were used in both samples), but from figure 3 it seems reasonable to conclude that average wing size across rep-



**Figure 2:** Average frequencies (with 95% confidence intervals) of standard gene arrangements after 1 (May 2002: *left panels*) and 2 years (April 2003: *right panels*) of thermal selection (*filled circles* = R1, *filled squares* = R2, *filled diamonds* = R3). Paired panels (left-right) are plotted at the same scale for easier contrasts. The small arrows in the left panels point to the corresponding initial frequency in the founding population derived from Puerto Montt (Chile). Confidence intervals were calculated by using the bootstrap percentile method after 10,000 bootstrap samples (Efron and Tibshirani 1993).

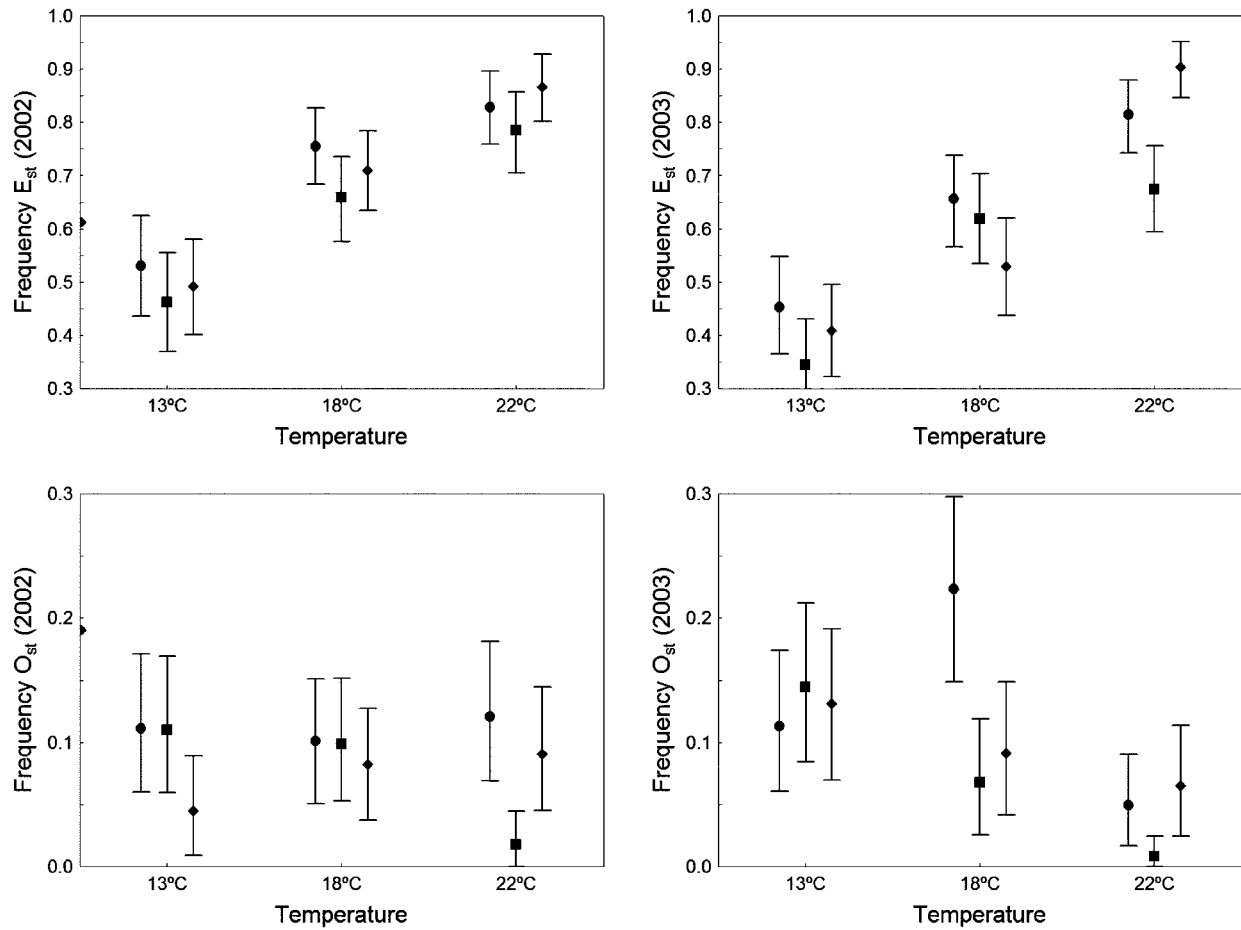


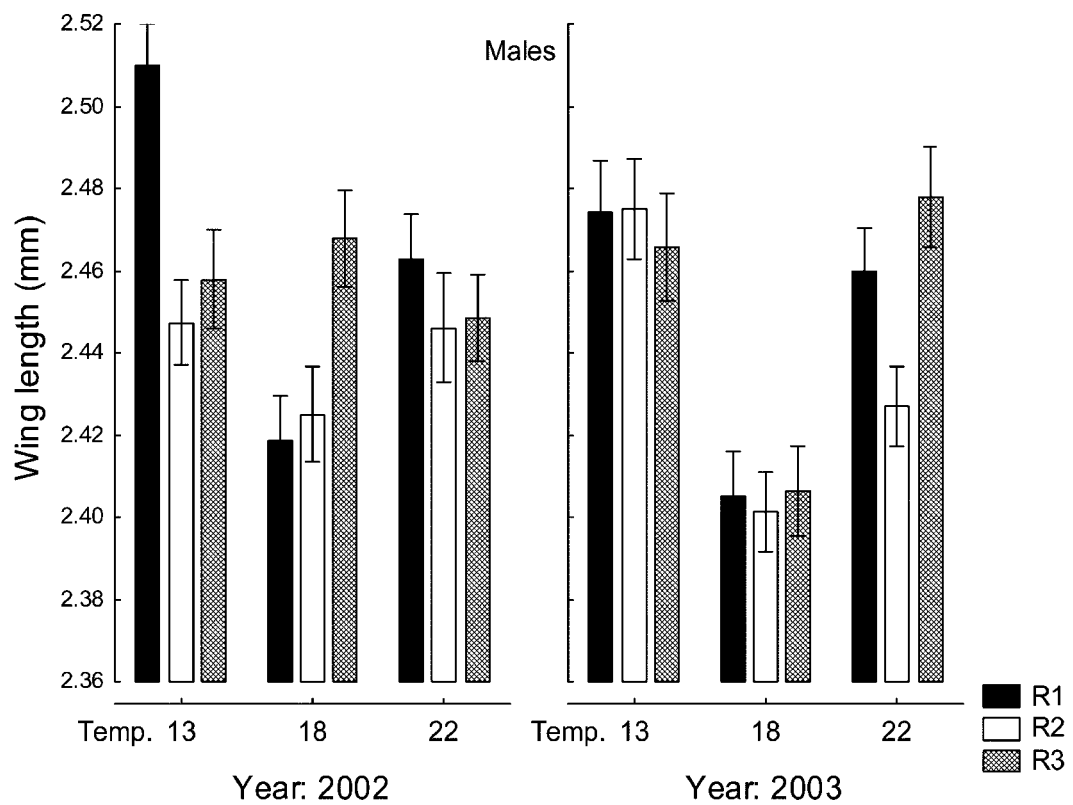
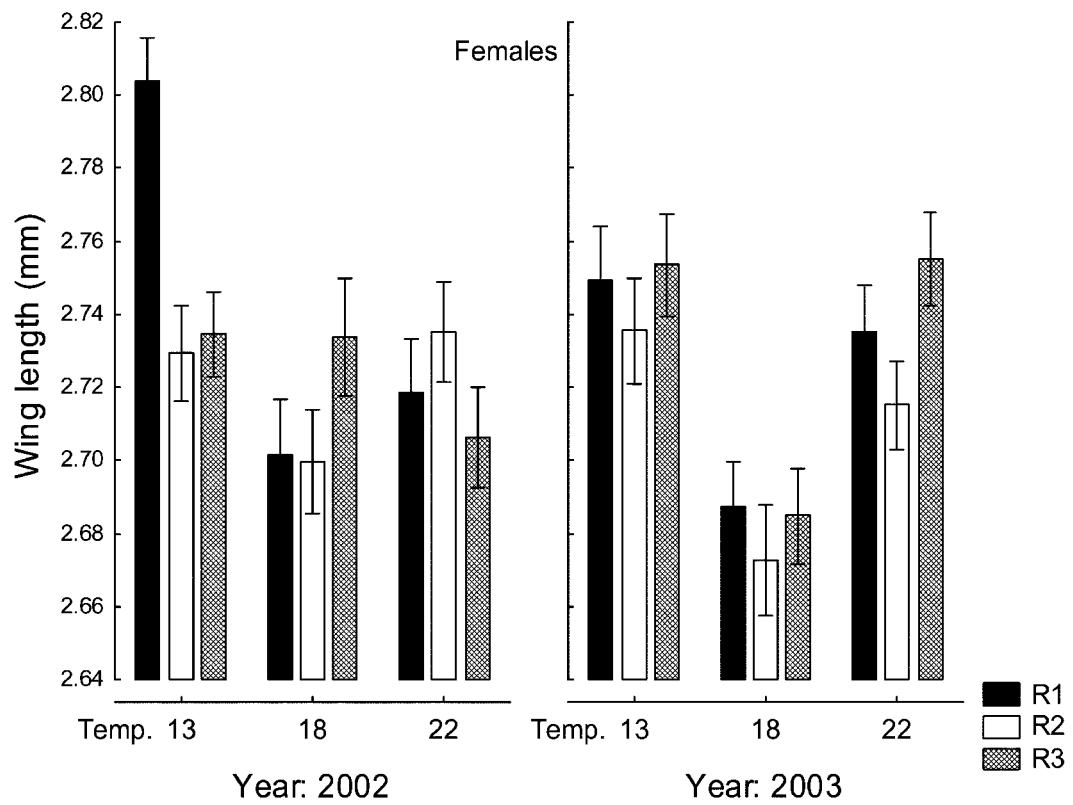
Figure 2 (Continued)

licated populations within each thermal regime has decreased at 18°C and remained quite unchanged at 13°C and 22°C. In fact, two-way ANOVAs with temperature and year as the main fixed effects, and replicate populations nested within temperature, indicated a highly significant temperature  $\times$  year interaction effect (females:  $F = 15.75$ ,  $df = 2, 1,783$ ,  $P < .001$ ; males:  $F = 17.66$ ,  $df = 2, 2,096$ ,  $P < .001$ ). However, orthogonal contrasts showed that adult flies from the populations at 13°C (females May 2002 [2.7560 mm] vs. April 2003 [2.7462],  $P = .094$ ; males May 2002 [2.4721] vs. April 2003 [2.4717],  $P = .942$ ) and 22°C (females May 2002 [2.7200 mm] vs. April 2003 [2.7352],  $P = .008$ ; males May 2002 [2.4526 mm] vs. April 2003 [2.4548],  $P = .589$ ) had indeed about the same average WL in both samples (most remarkable in the case of males). If anything has happened between the two samples at the two extreme temperatures, then female's size seems to have decreased at the lowest temperature and increased at the highest one.

**Cell Size and Cell Number Index.** The random sample of 50 wings per sex and population used to estimate the cellular components of body size differentiation showed the same pattern for WL as that plotted in figure 3 for the whole sample (females:  $F = 22.44$ ,  $df = 2, 6$ ,  $P = .002$ ; males:  $F = 13.43$ ,  $df = 2, 6$ ,  $P = .006$ ). Two-level nested ANOVAs of cell area did not detect statistical significant differences among temperatures (females:  $F = 3.00$ ,  $df = 2, 6$ ,  $P = .125$ ; males:  $F = 1.36$ ,  $df = 2, 6$ ,  $P = .326$ ; see fig. 4A), which at first glance would suggest that the aforementioned reduction in wing size at the optimum thermal selection regime was mainly brought about by a decrease in the number of cells in the wing blade. However, the average number of cells (cell number index) was also roughly the same across thermal treatments (females:  $F = 0.94$ ,  $df = 2, 6$ ,  $P = .441$ ; males:  $F = 2.29$ ,  $df = 2, 6$ ,  $P = .182$ ; see fig. 4B).

A principal component (PC) analysis for cell size/cell





number clearly defined an inverse relationship between both variables. Female loadings are

$$PC1 = \begin{pmatrix} -2.77 \times 10^{-3} \\ 1 \end{pmatrix},$$

$$PC2 = \begin{pmatrix} 1 \\ 2.77 \times 10^{-3} \end{pmatrix}.$$

Male loadings are

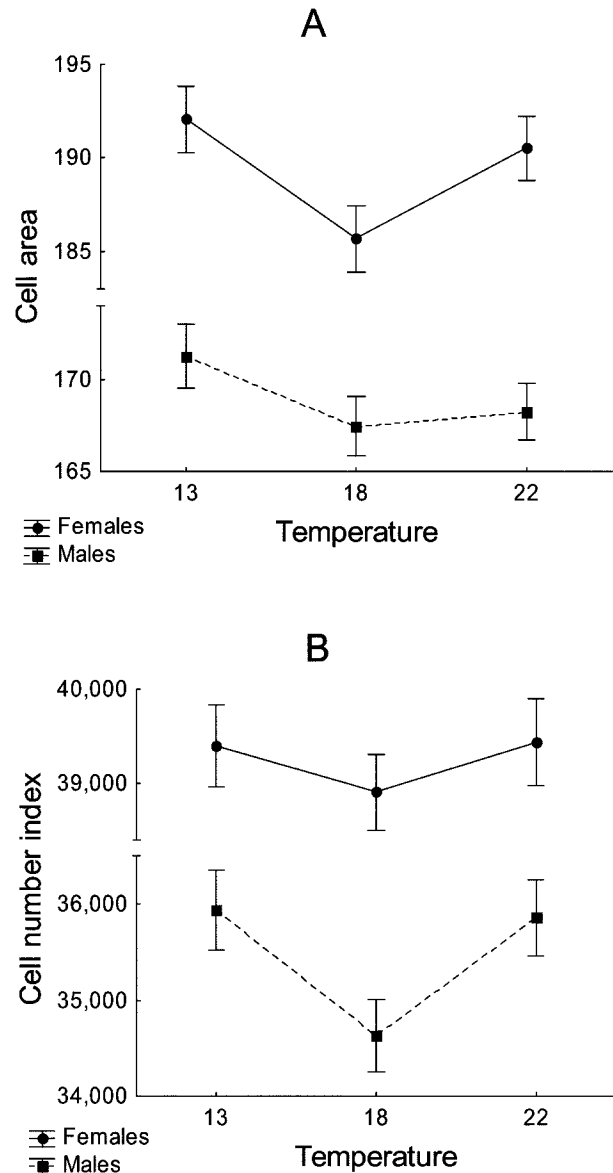
$$PC1 = \begin{pmatrix} -2.45 \times 10^{-3} \\ 1 \end{pmatrix},$$

$$PC2 = \begin{pmatrix} 1 \\ 2.45 \times 10^{-3} \end{pmatrix}.$$

Two-level nested ANOVAs on PC2 (or the computed  $z$  scores) detected statistically significant differences among temperatures (females:  $F = 10.27$ ,  $df = 2, 6$ ,  $P = .012$ ; males:  $F = 10.97$ ,  $df = 2, 6$ ,  $P = .010$ ). Overall, it seems that the ~2% reduction in wing size at the optimum temperature of 18°C was mediated by both cell area and cell number. However, the response in cell area was more important in females while the opposite appears to be true in males. In fact, a two-way ANOVA for PC2 with temperature and sex as the main fixed effects, and replicate populations nested within temperature, rendered a statistically significant temperature  $\times$  sex interaction ( $F = 3.02$ ,  $df = 2, 888$ ,  $P = .049$ ).

#### *Relationship between Chromosomal Polymorphism and Wing Size and Shape Index*

Sample sizes from the thermal stocks amount to more than 2,000 males for which we have simultaneous information on chromosome arrangements and wing dimensions. Three-way ANOVAs for WL, with temperature, year, and gene arrangement as the main fixed effects, and replicate populations nested within temperature are shown in table 1. The relevant effects here are those for gene arrangement and all its interaction terms. In no case were these interaction terms statistically significant, thus suggesting quite consistent results across thermal regimes and samples for the effects of chromosome arrangements on wing length.



**Figure 4:** Means and 95% confidence intervals for (A) cell area (values in  $\mu\text{m}^2$ ) and (B) cell number index from the thermal selection stocks when reared at 18°C under uncrowded conditions. To enhance visibility, all replicate populations within temperature have been pooled.

**Figure 3:** Means and 95% confidence intervals for wing length of *Drosophila subobscura* females (top) and males (bottom) from the thermal selection stocks when reared at 18°C under uncrowded conditions. Data from 2002 had been previously published (Santos et al. 2004) and are plotted here for comparisons. Female sample sizes in 2003 were the following: 13°C, R1 = 98, R2 = 100, R3 = 100; 18°C, R1 = 100, R2 = 100, R3 = 100; 22°C, R1 = 98, R2 = 99, R3 = 100. Male sample sizes were the following: 13°C, R1 = 115, R2 = 116, R3 = 111; 18°C, R1 = 113, R2 = 115, R3 = 117; 22°C, R1 = 114, R2 = 120, R3 = 119.

**Table 1:** Three-way ANOVAs for the wing length of *Drosophila subobscura* males

Source	Chromosome A		Chromosome J		Chromosome U		Chromosome E		Chromosome O <sup>a</sup>	
	df	MS	df	MS	df	MS	df	MS	df	MS
Temperature (T)	2	5.024*	2	4.076*	2	7.015*	2	3.602*	2	.816*
Replicate C (T)	6	.975***	6	1.007***	6	1.017***	6	.979***	6	1.012***
Year (Y)	1	.613**	1	.391*	1	1.154***	1	.558**	1	.065 NS
Arrangement (A)	1	.709**	1	.002 NS	2	.084 NS	3	.773***	5	.244**
T × Y	2	.584***	2	.551***	2	1.196***	2	.417**	2	.252*
T × A	2	.152 NS	2	.119 NS	4	.092 NS	6	.056 NS	10	.033 NS
Y × A	1	.003 NS	1	.014 NS	2	.134 NS	3	.036 NS	5	.044 NS
T × Y × A	2	.000 NS	2	.017 NS	4	.121 NS	6	.065 NS	10	.097 NS
Error	2,009	.067	2,073	.067	2,065	.067	2,061	.066	2,048	.066

Note: ANOVAs performed to test for biometrical effects of the polymorphic gene arrangements on the major five acrocentric chromosomes. WL as  $\log_e(L1 + L2)$  in pixels. MS  $\times 10^{-2}$ ; C = nested in; NS = not significant at  $P > .05$ .

<sup>a</sup> The recombination-derived O<sub>7</sub> gene arrangement was excluded from the analysis.

\*  $P < .05$ .

\*\*  $P < .01$ .

\*\*\*  $P < .001$ .

Polymorphic inversions on chromosomes A, E, and O seem to have a statistically significant effect on body size with the following patterns (after Scheffé post hoc tests where appropriate):  $A_{st} < A_2$ ,  $E_{1+2+9+3} < E_{st} = E_{1+2+9+12}$ ,  $O_{3+4+8} < O_{3+4+2}$ . The only case where a standard gene arrangement was associated with a larger body size when compared to a nonstandard one was for chromosome E, but arrangement  $E_{1+2+9+3}$  shows a quite flat relationship with latitude in New World populations (see Balanyà et al. 2003).

In the May 2002 sample, we had detected a significant and consistent (across thermal selection regimes) reduction of the wing shape index L1/WL with increasing standard dose, that is, with the number of standard gene arrangements carried out by a male, which ranges from 0 to 5 (Santos et al. 2004). A three-way ANOVA for L1/WL (as  $\log_e[L1/WL]$ ), with temperature, year, and standard dose (0, 1, 2, and  $\geq 3$ ) as the main fixed effects, and replicate populations nested within temperature revealed that none of the interaction terms involving the standard dose were statistically significant (results not shown). Therefore, the previously reported trend of a relative shortening of the basal length of longitudinal vein IV with an increase of the standard dose in *D. subobscura* flies from the New World is highly reliable.

### Discussion

Replicate thermal selection stocks of *Drosophila subobscura* flies derived from the estimated epicenter of the original New World invasion and maintained on a discrete generation, controlled larval crowding regime showed, first, a lower chromosomal diversity at the warmest and sub-optimal temperature; second, a quick and consistent shift

in gene arrangement frequencies in response to thermal selection regime; third, an evolutionary decrease in wing size—mediated by both cell area and cell number—at the optimum temperature of the species; fourth, no positive relationship between wing size and the standard gene arrangements on the five major acrocentric chromosomes; and fifth, a shortening of the basal length of longitudinal vein IV relative to its total length (i.e., wing shape index) with increasing standard dose. In the following, we discuss these findings in relation to patterns found in natural populations and results from earlier laboratory thermal selection experiments with various *Drosophila* species.

### Patterns of Chromosomal Diversity

At first glance, the negative relationship we found between chromosomal diversity and the evolutionary effect of temperature is against the geographic distribution of 1 – IFR in the Old World. This index decreases in a south-eastern (Asia Minor, Balkans)—north-northwestern (Scandinavia, Scotland) direction from 0.30 to 0.10, but with some spots of relatively high diversity (0.20) in North-eastern Spain (see Krimbas 1992, p. 194). This Palearctic geographic pattern (traditionally interpreted in terms of ecological “centrality” or “marginality”; see Krimbas and Loukas 1980), however, might bear little relationship with temperature adaptation and could be simply explained as a postglacial population expansion from a Balkan refugium. Actually, from a number of European case studies (e.g., the common meadow grasshopper of Europe, *Chorthippus parallelus*), it appears that the Balkans—as well as Italy, Spain, and Turkey, which also contained refugia for several species—were a source for all species in the east and for many species in the west (Hewitt 2000, 2001).

Particularly, the Southern Balkan peninsula harbors some *D. subobscura* gene arrangements specific for that refugium (e.g.,  $E_8$ ,  $U_{1+2+6}$ ) in high frequencies (Menozzi and Krimbas 1992). Thus, the standing geographic patterns of distribution in the Old World could only mirror historical processes and do not easily allow inferring current adaptation.

#### *Inversion Clines and Laboratory Cage Results*

The results of inversion frequency shifts after the New World invasion by *D. subobscura* were clear: clines fully developed within the first 5 years or so after colonization, and evolution was predictable (Balanyà et al. 2003). However, what remains largely unknown is whether such patterns were driven primarily by abiotic or biotic processes.

Because our thermal stocks were initiated from an ancestral population sampled at the estimated Chilean epicenter of the original New World invasion, these results should be more appropriately contrasted against the New World latitudinal clines summarized in Balanyà et al. (2003). 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  $O_{st}$ ). For chromosome U, no trends were detected in the thermal stocks, while clear latitudinal clines are evident in New World populations with  $U_{st}$  increasing and  $U_{1+2+8}$  decreasing with increasing latitude. On the other hand, for chromosome E, the trends observed in the thermal stocks were just opposite of those observed in nature, where arrangement  $E_{st}$  sharply increased in frequency with latitude soon after colonization. What seems to be quite coherent, however, is that by and large the chromosomal inversion polymorphism quickly responded to laboratory temperature: several gene arrangements with no latitudinal trends also showed a flat relationship with experimental temperature.

Certainly, laboratory experiments are not the best way to reconstruct natural clines. Our experimental controlled conditions obviously do not mirror the nutritional status or the daily and seasonal ranges in temperature experienced by flies in the field and might have been unsuccessful at identifying the real impact of temperature on polymorphic inversions (see Mitrovski and Hoffmann 2001; Hoffman et al. 2003). With these caveats in mind, however, we could tentatively conclude that the natural patterns do not seem to be generally caused by a direct effect of temperature. Previous laboratory cage experiments (summarized in Krimbas 1993) had also reached similar conclusions, but results were usually inconsistent because lines were started with different and often genetically homogeneous base populations. Inversion-related selective dis-

persal (see Gosteli 1991), karyotype by environment interactions of selection in wild populations, and/or selective agents correlated with latitude may work together to generate clinal patterns in New World colonizing populations.

#### *Clinal Variation of Body Size and Inversion Polymorphism*

In contrast to what had been observed for chromosome arrangements, wing size latitudinal clines were not evident in either North or South America about 1 decade after the introduction of *D. subobscura* (Budnik et al. 1991; Pegueroles et al. 1995), but it took ~20 years for them to evolve on both continents (Huey et al. 2000; Gilchrist et al. 2001, 2004; Calboli et al. 2003a). This plain fact already suggests that body size in New World populations was likely unrelated to those gene arrangements implicated in the latitudinal clines, which is against some claims based on European samples that point to a positive association between wing size and standard gene arrangement dose (Prevosti 1967; Orengo and Prevosti 2002; but see Krimbas 1967).

Correlations between body size and polymorphic inversions have certainly been observed for different *Drosophila* species. Thus, in *Drosophila buzzatii*, gene arrangements on chromosome 2 have a worldwide consistent biometrical effect on body size (Betrán et al. 1998), and in *Drosophila melanogaster*, some cosmopolitan inversions on the major autosomal arms have also been reported to affect size-related traits (e.g., Stalker 1980; van Delden and Kamping 1991). Indeed, the recently published quantitative trait loci (QTL) mapping results by Gockel et al. (2002) and Calboli et al. (2003b) show that 77% of the underlying latitudinal clinal variance of *D. melanogaster* body size in Australia and South America (see also Gockel et al. 2001) is explained by the third chromosome; additionally, the highest QTL peaks, explaining almost completely the variance due to that chromosome, maps on the inversion *In(3R)Payne*, which itself shows clinal variation (see Knibb et al. 1981; Knibb 1982; Weeks et al. 2002). Similar interactions with inversion *In(2L)t* in quantitative-trait clines from Australia and the west coast of South America are also quite likely (see James et al. 1995; van't Land et al. 2000). These results forcefully stress the difficulty of disentangling the effects of *Drosophila* polymorphic inversions on the clinal variation in quantitative traits (e.g., body size). Our present data, however, clearly show that New World latitudinal clines for polymorphic inversions and body size (but not wing shape; see Santos et al. 2004 and above) in *D. subobscura* are uncoupled. Obviously, this finding does not rule out the possibility that the long-standing body size cline in the Old World (Prevosti 1955; Misra and Reeve 1964; Pfriem 1983; Pegueroles et al. 1995) could certainly be correlated with the clines in chromo-

somal arrangement frequencies, and, therefore, the lack of correlation in samples from the New World might be a by-product of a founder effect that occurred during the colonization. In any case, it is worth mentioning that the autosomes that seem to have an effect on body size in *D. subobscura* (E and O) are homologous to those that have been consistently implicated in the control of body size in *D. melanogaster* latitudinal clines (2R and 3R, respectively; Gockel et al. 2002; Calboli et al. 2003b) and in *D. buzzatii* (chromosome 2 is homologous to chromosome O).

#### *Laboratory Thermal Evolution of Body Size*

Warm-adapted populations (22°C) were not smaller than their cold-adapted (13°C) counterparts and had about the same cell size (fig. 4A). It could be claimed that this result might be due to the relatively short duration of the selection experiment because thermal evolution of body size in *Drosophila* had been previously shown in populations kept at various temperatures for long periods of time (>4 years; Anderson 1973; Cavicchi et al. 1989; Partridge et al. 1994a). However, the comparative body size reduction of ~2% observed at the optimum temperature of 18°C (fig. 3; an incipient but nonsignificant decrease was already observed in the first sample) shows that an evolutionary response of wing size has already occurred. This reduction was mediated by both cell area and cell number. A quite general result of laboratory thermal selection experiments is that differences in wing area are entirely a consequence of cell size divergence (Robertson 1959; Cavicchi et al. 1985; Partridge et al. 1994a). However, our results seem to confirm Partridge et al.'s (1999) findings where a correlated response to decreasing size was mediated by both cell area and cell number.

As argued earlier, a common oversight in thermal selection experiments was the lack of control of larval culture density across temperatures. A crowded environment can cause high mortality at the egg, larval, and pupal stages. As the food is consumed, harmful waste products also accumulate (Borash et al. 1998), suggesting that growth rate will decline over time. Selection is expected to favor more competitive larva, which would likely cause a faster deterioration of the culture, as suggested by the results of Joshi and Mueller (1996) and Santos et al. (1997), showing that populations adapted to larval crowding consumed more food but used it less efficiently. Therefore, the additional results that cold-adapted populations have a greater efficiency of conversion of food to biomass and reduced competitive ability (Partridge et al. 1994b; Neat et al. 1995) could be explained to some extent by the common observation that populations of *D. melanogaster* stabilize at lower densities when maintained at lower tem-

peratures. Although there is no clear theoretical prediction concerning the evolution of body size (Mueller 1988), the outcomes might be dependent on the larval crowding regime. Thus, populations adapted to very high levels of larval crowding show about the same size at eclosion when assayed at low densities than their control counterparts (Santos et al. 1997). However, selection at moderate larval densities may result in an evolutionary decline of body size (Roper et al. 1996). Wolf (2003) has recently shown that a considerable portion of the genetic variation for body size in *Drosophila* may result from interactions among individuals and has also suggested that genetic variation for competitive ability appears as genetic variation for body size. Furthermore, maintenance at high larval densities led to the evolution of greater population growth rates at high density (Mueller and Ayala 1981), and there is suggestive evidence that adaptations to larval versus adult crowding trade off in *D. melanogaster* (Joshi et al. 1998; Borash and Ho 2001). Overall, the overwhelming evidence showing that density-dependent natural selection affects important life-history characteristics (reviewed in Mueller 1997) certainly sets important hurdles on our understanding of thermal adaptation per se in *Drosophila*.

As far as we are aware, there are only three previous laboratory thermal selection experiments carried out in *D. melanogaster* that have controlled for larval densities. Cavicchi et al. (1985, 1989) set up three populations reared at three different temperatures and maintained them for more than 4 years by placing 10 random pairs in each of four bottles containing abundant food. Unfortunately, the number of breeding adults per population was quite low, and the lines were not replicated, so genetic drift cannot be ruled out as the cause of the population divergence (notice from fig. 3 the large variation in body size among our replicates). More recently, Bochdanovits and de Jong (2003a) have analyzed the interaction between temperature and food quality regimes by setting four experimental evolution lines of *D. melanogaster* at four different combinations. They found that thermal evolution of body size interacts with the food quality regime; however, lines were kept with a relatively low number of breeding adults and were not replicated, so it would be highly desirable to repeat those experiments. Last, W. J. Kennington and J. Gockel (unpublished results) did not find any size differentiation after 55 generations of thermal selection under standardized larval crowding. Even though the interplay between temperature and larval density has never been critically addressed, we think that two clear conclusions emerge when all the information is taken together: first, larval crowding seems to play an important role in the establishment of body size differences in thermal selection experiments and may also play a part in the establishment of body size clines (see also Partridge and French 1996);

second, it is not necessarily true that adaptation at low temperature per se invariably results in the evolution of a larger body size in *Drosophila*. New experiments manipulating both temperature and larval density are highly needed.

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