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Swift laboratory thermal evolution of wing shape (but not size) in *Drosophila subobscura* and its relationship with chromosomal inversion polymorphism

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Abstract

Latitudinal clinal variation in wing size and shape has evolved in North American populations of *Drosophila subobscura* within about 20 years since colonization. While the size cline is consistent to that found in original European populations (and globally in other *Drosophila* species), different parts of the wing have evolved on the two continents. This clearly suggests that 'chance and necessity' are simultaneously playing their roles in the process of adaptation. We report here rapid and consistent thermal evolution of wing shape (but not size) that apparently is at odds with that suggestion. Three replicated populations of *D. subobscura* derived from an outbred stock at Puerto Montt (Chile) were kept at each of three temperatures (13, 18 and 22 °C) for 1 year and have diverged for 27 generations at most. We used the methods of geometric morphometrics to study wing shape variation in both females and males from the thermal stocks, and rates of genetic divergence for wing shape were found to be as fast or even faster than those previously estimated for wing size on a continental scale. These shape changes did not follow a neat linear trend with temperature, and are associated with localized shifts of particular landmarks with some differences between sexes. Wing shape variables were found to differ in response to male genetic constitution for polymorphic chromosomal inversions, which strongly suggests that changes in gene arrangement frequencies as a response to temperature underlie the correlated changes in wing shape because of gene-inversion linkage disequilibria. In fact, we also suggest that the shape cline in North America likely predated the size cline and is consistent with the quite different evolutionary rates between inversion and size clines. These findings cast strong doubts on the supposed 'unpredictability' of the geographical cline for wing traits in *D. subobscura* North American colonizing populations.

Introduction

Patterns of morphological variation mostly involving size-related dimensions across latitudinal/altitudinal gradients are often interpreted in relation to climatic conditions, mainly temperature. In endotherms this is exemplified by Bergmann's rule: 'the smaller-sized geographical races of a species are found in the warmer parts of the range, the larger-sized races in the cooler districts' (Mayr, 1942). Geographical clines in body size, with genetically larger individuals derived from higher latitudes, have also been documented in a number of ectothermic animals, particularly insects from the genus *Drosophila* (e.g. Stalker & Carson, 1947; Prevosti, 1955; David *et al.*, 1977; Coyne & Beecham, 1987; Pegueroles *et al.*, 1995; James *et al.*, 1997; van't Land *et al.*, 1999; Huey *et al.*, 2000). Is there a Bergmann's rule in

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ectotherms (cf. Mousseau, 1997; Partridge & Coyne, 1997; Van Voorhies, 1997)? Fitness costs related to the effects of surface/volume ratio on heat loss are assumed to underlie the rule, but it is obvious that size clines in ectotherms warrant a different explanation because small insects adopt ambient temperature almost instantaneously (Stevenson, 1985).

Although temperature is not the only factor that varies with latitude, laboratory studies carried out with a number of *Drosophila* species have repeatedly observed thermal selection on body dimensions that goes in the predicted direction according to the size clines (Anderson, 1966; Powell, 1974; Cavicchi *et al.*, 1985, 1989; Partridge *et al.*, 1994a). There are, however, some potentially important shortcomings with these experiments. Thus, no replicated populations were kept in Powell's or Cavicchi's *et al.* studies and, hence, the 'random-walk' hypothesis (which claims that evolutionary rates generally do not exist) cannot be discarded (see Bookstein, 1991, pp. 393–398). However, Anderson's and Partridge's *et al.* thermal stocks were replicated but flies were maintained in population cages by regularly introducing bottles with fresh food and removing them after several weeks, a routine that does not allow control of larval densities. Crowding conditions differ between population cages maintained at different temperatures, with higher larval densities in warmer environments (L. Partridge, pers. comm., 1998). There is conflicting evidence about the importance of larval density on the evolutionary responses of body size in *Drosophila* (cf. Roper *et al.*, 1996; Santos *et al.*, 1997); however, it is well established that harmful waste products accumulate in crowded cultures (Borash *et al.*, 1998, 2000) and many of the adaptations to different levels of larval crowding (Joshi & Mueller, 1988, 1993; Guo *et al.*, 1991; Mueller *et al.*, 1993; Borash *et al.*, 1998) involve changes in larval behaviour and physiology that may impinge on other phases of the life cycle (see e.g. Joshi & Mueller, 1996; Santos, 1996; Santos *et al.*, 1997; Houle & Rowe, 2003). Therefore, although the previous studies – as well as those that have also reported evolutionary responses in other fitness-related traits in the thermal stocks (Huey *et al.*, 1991; Partridge *et al.*, 1994b; James & Partridge, 1995; Azevedo *et al.*, 1996) – do demonstrate rapid adaptations, it is by no means obvious that temperature is the main factor or that body size is the target of selection (see Bochdanovits & De Jong, 2003).

The historically Paleartic species *D. subobscura* provides a suitable model system to study the dynamics of clinal variation and the biological effects of the current greenhouse-induced increase in world temperatures (Houghton *et al.*, 2001) for a number of reasons. First, there is an extensive amount of information on the geographical distribution of chromosomal arrangements in this particularly inversion-rich species, with the so-called standard arrangements in the five (out of six considering the dot chromosome) acrocentric chromosomes increasing in frequency with latitude in European populations (Krimbas & Loukas, 1980; Menozzi & Krimbas, 1992). Most important, similar clines quickly developed (within about 7 years or ~35 generations) after the double colonization of South and North America by the species (Prevosti *et al.*, 1985, 1988), strongly supporting the hypothesis that environmental latitudinal gradients are responsible for the clines. Further support comes from several independent observations showing within-population long-term directional trends in the inversion polymorphism that correlated with expectations from the latitudinal clines (Orengo & Prevosti, 1996; Solé *et al.*, 2002), although a putative critical role played by a shift in temperatures could only be ascribed to those trends tracked for the gene arrangements of chromosome O for which relatively long time-series matching both the inversion frequencies and the variation in temperatures exist (Rodríguez-Trelles *et al.*, 1996; Rodríguez-Trelles & Rodríguez, 1998).

Secondly, parallel body size clines as the long-standing ones in native European populations (Prevosti, 1955; Misra & Reeve, 1964; Pfriem, 1983; Pegueroles *et al.*, 1995) appeared in New World populations too, although it took about 20 years after the colonization for the clines to build up (Huey *et al.*, 2000; Gilchrist *et al.*, 2001). Interestingly, the size clines are not isometric in the sense that the relative contribution of two portions of longitudinal vein IV used to measure wing length (WL) also changed with latitude but in an opposite way according to the northern hemisphere continent (i.e. Europe vs. North America). On the basis of previous studies in *D. melanogaster* (Gilchrist *et al.*, 2000) it was hypothesized that the wing shape variation in *D. subobscura* may simply represent drift around an optimum. Consistent with this idea, recent biometric and quantitative trait loci (QTL) analyses suggest that wing size and shape have a contrasting genetic architecture; the former likely being subjected to directional selection and the latter to optimizing selection and regulated largely independently of wing size, with up to 50 loci throughout the *D. melanogaster* genome having a significant and generally additive effect on wing shape as well as minor pleiotropic effects on fitness (Weber *et al.*, 1999, 2001; Zimmerman *et al.*, 2000; Gilchrist & Partridge, 2001). Birdsall *et al.* (2000) used the method of relative warps (Bookstein, 1991) to study wing shape variation in 12 inbred lines from *D. melanogaster* at 18 and 25 °C. They found that the two rearing temperatures caused differences in wing area of up to 20%, but wing shape seemed to be independent of sex and temperature effects on cell growth and density. This is important because the cellular basis of the body size cline for *D. subobscura* in North America (latitudinal variation in cell area) is different from that in Europe and South America clines (latitudinal variation in cell number; Calboli *et al.*, 2003). Therefore, the opposite latitudinal gradients for wing shape (Huey *et al.*, 2000)

do not seem to be related to the cellular bases of the clines, as suggested by the work of Birdsall *et al.* (2000).

Third and finally, Orengo & Prevosti (2002) recently presented evidence for a positive relationship between wing size and standard gene arrangement dose, an expected trend according to the latitudinal clines. However, they used two samples of wild-caught *D. subobscura* males and the likely presence of nongenetic effects on body size precludes any firm conclusion. In addition, the different evolutionary rates observed in colonizing populations when comparing chromosomal polymorphism and wing size (see above) is somewhat puzzling from that putative genetic connection. Further work is clearly needed.

We have developed a set of three replicated *D. subobscura* populations kept at three temperatures (13, 18 and 22 °C) to study the short- and long-term effects of thermal selection on chromosomal inversion polymorphism and wing size and shape. A large stock from Puerto Montt (Chile) was chosen as the base population because (1) the species was detected for the first time in America at this locality in February 1978 (Brncic *et al.*, 1981); (2) the stock harboured all polymorphic chromosome arrangements involved in the New World latitudinal clines (Prevosti *et al.*, 1988); and (3) the introduction of the species into South and North America (first detected in Port Townsend, Washington, in 1982; Beckenbach & Prevosti, 1986) was the result of a single colonizing event from a Paleartic population (Mestres *et al.*, 1992), which provides a unique opportunity to empirically test how replicated clinal patterns in nature relate to temperature. Our rearing protocol allows controlling for larval densities, thus minimizing those potentially spurious correlated responses that may arise due to other factors not related to thermal selection. Here we report the initial results showing no size differences according to thermal regimes, a probably unsurprising finding for populations that have diverged for 27 generations at most. However, the analyses of wing shape by using the shape index in Huey *et al.* (2000) and the framework of geometric morphometrics (Bookstein, 1991; Dryden & Mardia, 1998) revealed consistent and significant differences among temperatures, a somewhat unexpected finding if shape clines were historically contingent as formerly sustained (Huey *et al.*, 2000). We discuss the results in the light of available information on *Drosophila* wing shape, and provide some empirical evidence as to suggest that the highly congruent results across replicated populations are related to gene-inversion linkage disequilibria.

Materials and methods

Thermal selection stocks

The *D. subobscura* populations originated from 93 isofemale strains derived from a large outbred stock collected by Dr J. Balanyà, Dr G. W. Gilchrist, Dr R. B. Huey and Dr M. Pascual in Puerto Montt (Chile; 41°28′S) in November 1999. The isofemale lines were kept in 90-mL bottles (~30–40 breeding adults/bottle) at 18 °C for more than 1 year (~16 generations) prior to the establishment of the thermal stocks and, hence, the experimental material had likely ceased to undergo rapid adaptation to laboratory conditions (Matos *et al.*, 2000). A large outbreeding population was founded in March 2001 by randomly dumping ~25 pairs of virgin flies from each isofemale line into three Plexiglas cages ($27 \times 21 \times 16$ cm^3) and maintained at 18 °C (12 : 12 light/dark cycle). A large number of eggs were collected from these cages, and emerging adults were randomly dumped into three new cages (18 °C; 12 : 12 light/dark cycle). Eggs were sampled from these cages over 10 consecutive days and placed in 130-mL bottles (~200–250 eggs per bottle) containing 50 mL of David's killed-yeast *Drosophila* medium (David, 1962). A total of 225 bottles were set up and randomly distributed into nine groups with 24 bottles each. Three groups were allocated at 15 °C (12 : 12 light/dark cycle), three at 18 °C (12 : 12 light/dark cycle) and three at 21 °C (12 : 12 light/dark cycle). Therefore, the three replicated thermal selection stocks were established in May 2001. The extra nine bottles were used to individually cross a random sample of emerging males to three to four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies in the initial populations. This strain is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu*) (Koske & 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_{3+4} (Lankinen & Pinsker, 1977). Whenever feasible, one F_1 female third-instar larva derived from each cross with the homozygous *ch-cu* stock was examined for its inversion loops in polythene chromosomes to determine the gene arrangements of one set of the chromosomes from the wild-type male.

After two generations of acclimatization the 15 °C stocks were transferred to the final temperature of 13 °C, and the 21 °C stocks to 22 °C. Previous laboratory observations (reviewed in Krimbas, 1993) indicate that optimal temperature for *D. subobscura* is approximately 18 °C, and that males can become sterile at 25 °C or even lower. Latitudinal clines for optimal temperatures are quite possible to occur; however, the three temperatures explored in our thermal selection stocks likely cover much of the physiologically tolerable range in this species.

All populations are maintained on a discrete generation, controlled larval crowding regime as follows. Prior to initiating a new generation, eclosed adults from the bottles are dumped into a Plexiglas cage and supplied with liberal amounts of food (two 90 mm Ø Petri dishes with *Drosophila* medium supplemented with active dried yeast) before egg collections. The number of breeding

adults per population is typically well over 1500 flies. Once females reach their peak of fecundity (~12–13 days after emergence at 13 °C, ~7–8 days at 18 °C and ~5–6 days at 22 °C) eggs are collected over a 7-day period at 13 °C, 5-day at 18 °C and 3-day at 22 °C, and placed in 130-mL bottles (~200–250 eggs per bottle) as previously described, with a total of 24 bottles per population. Generation times (eggs $t_n \rightarrow t_{n+1}$) are ~46 days at 13 °C, ~33 days at 18 °C and ~25 days at 22 °C. Consequently, the three sets of populations differ only in the temperature they experience (humidity was not strictly controlled but adult flies in the cages had continuing access to fresh and moist food).

In May 2002 (after nine generations at 13 °C, 12 generations at 18 °C, and 15 generations at 22 °C), samples from all populations were obtained by placing eggs into eight additional 130-mL bottles per population. These bottles were cultured at 18 °C and emerging adults were dumped into Plexiglas cages for egg collections. Eggs for the experiment were collected over a 48-h period by placing Petri dishes containing nonnutritive agar with a generous smear of live yeast in the cages. Larval density was controlled during culturing (100–110 eggs per bottle), and a total of 12 bottles per population were placed at 18 °C on the same incubator shelf. Therefore, the experiment was designed so that the parents of sampled flies had also been reared at the same temperature, to control for the possibility of nongenetic parental effects on offspring size (Crill *et al.*, 1996). Emerging flies were separated by sex; females stored in Eppendorf tubes with a 3 : 1 mixture of alcohol and glycerol at 4 °C, and a sample of males (125–150 males per population randomly chosen from the 12 replicated bottles) were individually crossed in vials (2 × 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 as previously described. 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_2 anaesthesia, on flies not less than 6 h after eclosion.

Wing size and shape

Definitions

Morphometrics involves the quantitative study of form, and it is naturally understood that form consists of size and shape (Needham, 1950). An important contribution of geometric morphometrics is the clear definition of size and shape (Dryden & Mardia, 1998). Size is defined as any positive real-valued function from a landmark configuration (i.e. a set of points that can be precisely located) matrix X that satisfies the condition $g[aX] = ag[X]$ for any positive scalar a. The shape of a set of p landmark points is the geometrical information of the configuration of points that is invariant to translation, rotation and rescaling.

Wing measurements

We analysed here the wing size and shape of flies from each experimental population. Both wings were removed and mounted on microscope slides in DPX under coverslips from 100 females per population selected haphazardly from the 12 replicated bottles (see above), as well as from all males crossed to the *ch-cu* marker strain whenever information of their chromosomal arrangements was available (>100 males per population; see Table 1). All the data used here are from the left wings. Wing images were captured using a compound microscope (Zeiss Axioskop, Jena, Germany), with low power objective (2.5×) and attached video camera (Sony CCD-Iris, Tokyo, Japan), connected to a PC computer with MGI VideoWave software. Female wings were digitized

Table 1 Mean (±SD) of the basal (L1) and distal (L2) segments of wing longitudinal vein IV, and centroid size (in a normalized form; see Dryden & Mardia, 1998, p. 24) in *Drosophila subobscura* for each thermal regime and replicated population (values are given in millimetres; 1 mm = 144 pixels).

		Females				Males			
Temperature (°C)	Replicate	*n*	L1	L2	Centroid size	*n*	L1	L2	Centroid size
13	R1	100	1.521 ± 0.040	1.283 ± 0.035	1.014 ± 0.021	115	1.356 ± 0.040	1.154 ± 0.034	0.907 ± 0.019
	R2	100	1.480 ± 0.046	1.250 ± 0.037	0.986 ± 0.023	115	1.322 ± 0.040	1.125 ± 0.034	0.884 ± 0.020
	R3	100	1.479 ± 0.044	1.256 ± 0.036	0.989 ± 0.022	107	1.322 ± 0.046	1.136 ± 0.036	0.887 ± 0.022
	Total	300	1.493 ± 0.048	1.263 ± 0.038	0.996 ± 0.025	337	1.334 ± 0.045	1.138 ± 0.037	0.893 ± 0.023
18	R1	100	1.455 ± 0.052	1.247 ± 0.040	0.975 ± 0.027	134	1.297 ± 0.045	1.122 ± 0.033	0.873 ± 0.023
	R2	100	1.452 ± 0.050	1.248 ± 0.037	0.972 ± 0.027	128	1.295 ± 0.049	1.130 ± 0.033	0.873 ± 0.024
	R3	100	1.472 ± 0.055	1.262 ± 0.038	0.986 ± 0.030	132	1.326 ± 0.046	1.142 ± 0.035	0.889 ± 0.024
	Total	300	1.460 ± 0.053	1.252 ± 0.039	0.978 ± 0.028	394	1.306 ± 0.048	1.131 ± 0.035	0.878 ± 0.025
22	R1	100	1.472 ± 0.052	1.246 ± 0.039	0.985 ± 0.026	117	1.323 ± 0.041	1.139 ± 0.034	0.889 ± 0.021
	R2	100	1.478 ± 0.050	1.257 ± 0.037	0.990 ± 0.024	109	1.313 ± 0.049	1.133 ± 0.035	0.883 ± 0.025
	R3	100	1.462 ± 0.050	1.244 ± 0.038	0.979 ± 0.025	111	1.313 ± 0.042	1.136 ± 0.037	0.884 ± 0.019
	Total	300	1.471 ± 0.051	1.249 ± 0.038	0.984 ± 0.025	337	1.317 ± 0.044	1.136 ± 0.035	0.885 ± 0.022

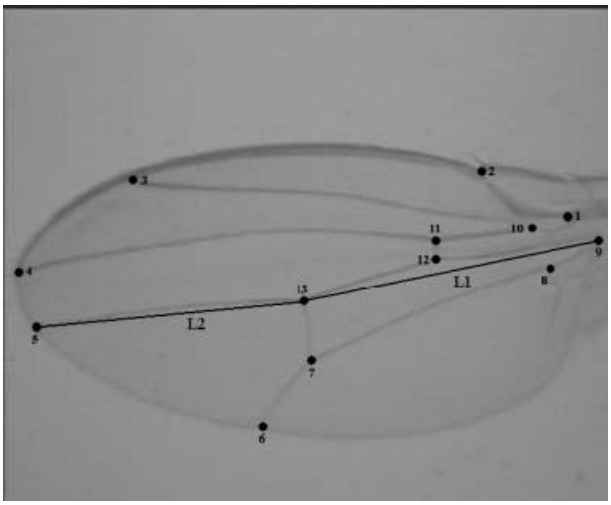

Fig. 1 Image of *Drosophila subobscura* left wing indicating the 13 landmarks used in this work. The lengths of the proximal (L1) and distal (L2) segments of longitudinal vein IV were calculated as the linear distance between landmarks 9 and13, and landmarks 13 and 5, respectively.

by M.S. and male wings by P.F.I., but all wings were measured by one of us (M.S.). Calibration of the optical system was checked at each session. *x* and *y* coordinates of 13 morphological landmarks (Fig. 1) were recorded for each wing using the image processing and analysis program for the IBM PC Scion Image (based on the NIH-Image for Macintosh and available at http://www.scioncorp.com). All landmarks are at the intersections of wing veins or at points where veins reach the wing margin and are easy to locate precisely, and can therefore be considered type 1 landmarks according to Bookstein (1991), pp. 63–67) or anatomical landmarks according to Dryden & Mardia (1998), p. 3). Using the original landmark coordinates we followed Robertson & Reeve (1952) and Prevosti (1955) by calculating WL as the combined lengths of the basal (i.e. the Euclidean distance between landmarks 9 and 13 and labelled as L1) and distal (Euclidean distance between landmarks 13 and 5 and labelled as L2) segments of longitudinal vein IV. These linear measurements have been used to study size and shape clines in *D. subobscura* (e.g. Pegueroles *et al.*, 1995; Huey *et al.*, 2000; Gilchrist *et al.*, 2001).

Procrustes analysis

Procrustes methods allow comparing configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit (Rohlf, 1990, 1999; Rohlf & Slice, 1990; Klingenberg & McIntyre, 1998). When several objects (e.g. wings) are fitted using Procrustes superimposition (as was done in the present work) the method has been called 'generalized Procrustes analysis' (GPA) (see Dryden & Mardia, 1998, pp. 44–47). There has been some controversy on the relative merits of GPA over alternative approaches, but Rohlf (2000, 2003) has shown that the mean shape in a population can be reliably estimated using GPA. In brief, the procedure can be described as follows (Rohlf & Slice, 1990; Bookstein, 1996): (1) shift the *x* and *y* coordinates to the origin (0, 0) and scale the configurations to unit centroid size (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); (2) rotate the configurations against a single reference specimen to achieve an optimal fit of corresponding landmarks; (3) a single overall consensus configuration is computed as the average coordinates of corresponding landmarks in the aligned configurations; (4) repeat steps 2 and 3 to minimize the sum of the squared distances between the landmarks of all objects in the sample and the corresponding landmarks of the consensus configurations. The final step was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden & Mardia (1998); see also Rohlf, 1999). [Rescaling the coordinates of each configuration by the scaling option, $1/\cos(\rho)$ (see Rohlf, 1999) would make very little difference, in the order of $\sim$0.0015 and $\sim$0.0004% of the shape variation in the female and male data sets, respectively]. The variation in the landmark coordinates that remains after Procrustes superimposition is a complete and nonredundant description of the variation in shape, and the usual linear multivariate methods focus on these coordinates (see below).

In this work we used MATLAB (V.5 and V.6; The MathWorks, Inc. 1998, 2002) for morphometric analyses, and results were checked with the 'tps' series of programs by J. F. Rohlf (available at http://life.bio.sunysb.edu/morph). Some helpful functions in morphometrics from the MATLAB toolboxes Res5 and Res6 developed by R. E. Strauss were also used (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm).

Statistical analyses

The unit of analysis here is the population, and the three replicated populations (R1, R2 and R3) of each thermal stock were treated as a random factor nested within experimental temperature (13, 18 and 22 °C), which was a fixed effect in the ANOVAS (see Sokal & Rohlf, 1981).

Allometry

To test for size effects on shape variation we carried out multivariate regressions of Procrustes coordinates on centroid size (Dryden & Mardia, 1998). These regressions generally accounted for around 4% of total Procrustes sums of squares; however, multivariate analyses using

the residuals of a regression on centroid size produced results that were qualitatively identical to those of the complete variation. Therefore, we only report the results of analyses of the total shape variation.

Procrustes ANOVA

As pointed out by Klingenberg & McIntyre (1998), calculation of Procrustes coordinates is based on the algebra of sums of squares, and the variance in the set of optimally aligned landmark configurations can be partitioned in a way analogous to the deviations from a grand mean in conventional ANOVA (Goodall, 1991). The coordinates of the Procrustes-aligned configurations are, therefore, amenable to the preceding two-level nested ANOVA model.

For each of the x and y coordinates of the aligned configurations a separated two-level nested ANOVA was run, and the resulting sums of squares for temperature, replicate and error in the Procrustes ANOVAS were obtained after summing the corresponding sums of squares across x and y coordinates of all landmarks. There are more degrees of freedom in Procrustes ANOVA than in conventional ANOVA (Goodall, 1991) because the squared deviations are summed over all the landmark coordinates. Therefore, the number of degrees of freedom is that for ordinary ANOVA times the shape dimension; namely, $2p - 4$ for two-dimensional coordinate data, where p is the number of landmarks.

To avoid making assumptions about the specific distribution of wing shapes around the mean landmark configuration we used permutation tests (Manly, 1997) for testing the statistical significance of ANOVA effects. Permutation tests also avoid the rather stringent statistical constraints of the covariance structure described by Goodall (1991) (see also Rohlf, 2000). For the two-level nested ANOVA model randomization is a two-stage process: (1) random permutations among subgroup (replicated populations) within group (experimental temperature) for the among-subgroup F-statistics; and (2) random permutations among subgroup and group for the among-group F-statistics. Each test used 10 000 random permutations of the observations.

Localized variation

In order to assess how much of shape variation was due to each landmark, we followed Klingenberg & McIntyre (1998) and decomposed the Procrustes mean squares for each effect in the two-level nested ANOVA model according to the landmarks. Thus, we summed x and y mean squares of each landmark separately and computed the variance components according to the expected mean squares (Sokal & Rohlf, 1981). Because the least-squares algorithm tends to spread variation from variable landmarks to the others, this approach should be taken cautiously if one or a few landmarks are much more variable than the rest (Chapman, 1990; Walker, 2000).

Shape variability

We used principal component analysis (PCA) (see e.g. Jolliffe, 1986) to investigate patterns of covariation in the positions of landmarks, which is a usual method in the context of shape analysis (Dryden & Mardia, 1998; Klingenberg & McIntyre, 1998; Klingenberg & Zaklan, 2000). The analyses must use covariance matrices of the coordinates of superimposed landmarks to avoid problems related to rotations of the coordinate system, and principal components coefficients can be presented graphically by drawing lines centred at the mean location of each landmark and ending at an arbitrary number of standard deviations away from that mean in the direction to which the landmark would shift.

The computer programs used for statistical data analyses were MATLAB (V.5 and V.6, The MathWorks, Inc. 1998, 2002), and the statistical software packages STATISTICA V.6 (StatSoft, Inc., 2003) and SPSS V.11 (SPSS Inc., 2001). They were run on a Pentium® 4 (1.60 GHz) PC-compatible.

Results

Wing size and shape index

The mean values for the basal (L1) and distal (L2) WLs and centroid sizes were calculated for females and males from each population (Table 1). Flies were considerably larger than the F_2 offspring from a wild sample collected in 1986 at the same locality of Puerto Montt and raised under similar conditions of food and temperature (see Pegueroles *et al.*, 1995). Females were approximately 11% bigger than males, and average sizes for the 13 °C thermal selection regime were slightly bigger than those at warmer temperatures. There was, however, substantial variation among replicated populations and the two-level nested ANOVAS did not detect any significant effect of thermal selection regimes on WL or centroid size. In addition, there was no indication of a linear trend between size and temperature (Table 2).

However, significant effects of thermal selection regimes (but not replicates) were observed for the length of L1 relative to total WL, and the patterns were similar in both sexes (Fig. 2). Nonlinear (deviation) effects were significant and, therefore, this shape index did not show a neat linear trend with temperature (Table 2). It is worth mentioning that *post hoc* comparisons for females only detected statistically significant differences when contrasting 18 °C vs. 13 °C or 22 °C; namely, the length of L1 relative to that observed at 18 °C significantly increased at the lowest and highest temperatures in females (but not in males where the shape index was significantly higher only at the lowest temperature; analyses not shown). When globally considered, these findings could be compatible with the contrasting patterns found in the native Paleartic populations (i.e. a positive correlation between the shape index and

Table 2 Two-level nested analyses of variance for wing length [WL: as $\log_e$(L1 + L2) in pixels; 1 mm = 144 pixels], centroid size (in pixels), and shape index (basal length/wing length) [as $\log_e$(L1/WL)]. The sums of squares for the fixed factor temperature were further decomposed to test for linear (regression) and nonlinear (deviation) effects.

		WL				Centroid size				L1/WL			
Source	d.f.	SS	MS	*F*	*P*	SS	MS	*F*	*P*	SS	MS	*F*	*P*
(a) Females													
Temperature	2	4.49×10^{-2}	2.24×10^{-2}	2.20	0.192	1063.5	531.7	2.33	0.179	7.09×10^{-3}	3.54×10^{-3}	18.23	0.003
Regression	1	2.89×10^{-2}	2.89×10^{-2}	2.84	0.143	498.8	498.8	2.18	0.190	0.94×10^{-3}	0.94×10^{-3}	4.83	0.070
Deviation	1	1.60×10^{-2}	1.60×10^{-2}	1.57	0.257	564.7	564.7	2.47	0.167	6.15×10^{-3}	6.15×10^{-3}	31.62	0.001
Replicate	6	6.11×10^{-2}	1.02×10^{-2}	15.47	<0.001	1370.4	228.4	17.56	<0.001	1.17×10^{-3}	0.19×10^{-3}	0.64	0.696
Error	891	0.59	6.58×10^{-4}			11592.9	13.0			0.27	0.30×10^{-3}		
(b) Males													
Temperature	2	3.73×10^{-2}	1.86×10^{-2}	1.46	0.304	789.7	394.9	1.94	0.223	8.77×10^{-3}	4.39×10^{-3}	6.79	0.029
Regression	1	1.29×10^{-2}	1.29×10^{-2}	1.01	0.353	238.6	238.6	1.17	0.320	4.62×10^{-3}	4.62×10^{-3}	7.14	0.037
Deviation	1	2.44×10^{-2}	2.44×10^{-2}	1.91	0.216	551.1	551.1	2.71	0.151	4.15×10^{-3}	4.15×10^{-3}	6.42	0.044
Replicate	6	7.67×10^{-2}	1.28×10^{-2}	19.78	<0.001	1220.7	203.4	20.12	<0.001	3.88×10^{-3}	0.65×10^{-3}	1.88	0.082
Error	1059	0.68	6.46×10^{-4}			10709.3	10.1			0.36	0.34×10^{-3}		

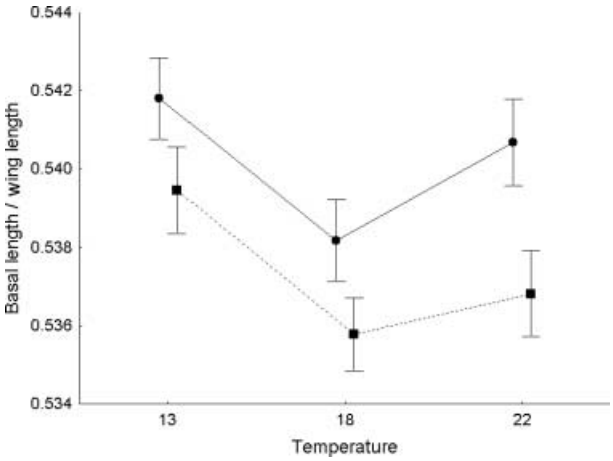

Fig. 2 Averages of the relative length (with 95% confidence intervals) of the basal portion of longitudinal vein IV (L1) to the total wing length (WL = L1 + L2) vs. experimental temperature for females (●) and males (■). All three replicated populations within each thermal regime were pooled because no statistically significant differences among replicates were detected in the two-way nested ANOVAS (see Table 2).

latitude) and in recently colonizing populations of North America (a negative correlation with latitude; Huey *et al.*, 2000).

Wing shape variability

The Procrustes two-level nested ANOVA for female wing shape variation led to the same previous conclusion regarding shape index L1/WL; namely, a significant and consistent (across replicated populations) shape variation was detected among thermal selection regimes but with a nonlinear trend for temperature (Table 3a. Notice that probabilities from the two-stage permutation test are higher than those from a 'true' *F*-statistics with the same degrees of freedom, which clearly indicates that the degrees of freedom in our Procrustes ANOVA are not independent and also suggests correlated landmark shifts.) However, no statistically significant differences were detected for males (Table 3b), which might suggest that they are lagging behind females. [Actually, when Procrustes distances were used as the dependent variable (which allows for global tests of shape differences), significant effects of thermal selection regimes were detected for both sexes (see below).] A differentiated pattern between sexes also emerged when variance components from the Procrustes ANOVAS were apportioned by landmarks. Thus, landmarks 1, 2 and 13 dominated for temperature effects in females but had relatively low amounts of variability in males. However, landmark 5 had the largest temperature effect in males (Fig. 3). To summarize, although the relative amounts of variation at each landmark vary markedly among the factors included in the two-way nested ANOVAS, temperature effects seem to be significant in both sexes for at least one landmark involved in the shape index L1/WL (as could be expected from the results in Table 2).

Principal component analyses were carried out for overall shape variation among individuals (within sexes) across the entire wing (i.e. from the covariance matrices of the coordinates of superimposed landmarks obtained from the 900 females and the 1068 males), as well as among thermal regimes (i.e. from the covariance matrices of the mean coordinates at each temperature). For individual variation the first two principal components explained at least 63 and 7% of the variability, respectively, and results were fairly consistent between sexes (Fig. 4). This high level of variability explained by a few PCs clearly suggests strong dependencies among landmarks and, hence, the isotropic model (which presumes that there is an equal amount of nondirectional variation

Table 3 Two-level nested analyses of variance for shape using Procrustes sums of squares as a measure of overall variation in shape. Statistical significance was computed after 10 000 random permutations (see text for details).

Source	d.f.	SS	MS	*F*	*P*
(a) Females					
Temperature	44	11.58×10^{-3}	2.63×10^{-4}	4.02	0.019
Regression	22	2.36×10^{-3}	1.07×10^{-4}	1.64	0.196
Deviation	22	9.22×10^{-3}	4.19×10^{-4}	6.40	0.007
Replicate	132	8.63×10^{-3}	0.65×10^{-4}	1.81	0.071
Error	19 602	696.81×10^{-3}	0.36×10^{-4}		
(b) Males					
Temperature	44	5.53×10^{-3}	1.26×10^{-4}	1.61	0.187
Regression	22	1.98×10^{-3}	0.90×10^{-4}	0.15	0.318
Deviation	22	3.55×10^{-3}	1.61×10^{-4}	2.07	0.113
Replicate	132	10.29×10^{-3}	0.78×10^{-4}	2.02	0.016
Error	23 298	899.41×10^{-3}	0.39×10^{-4}		

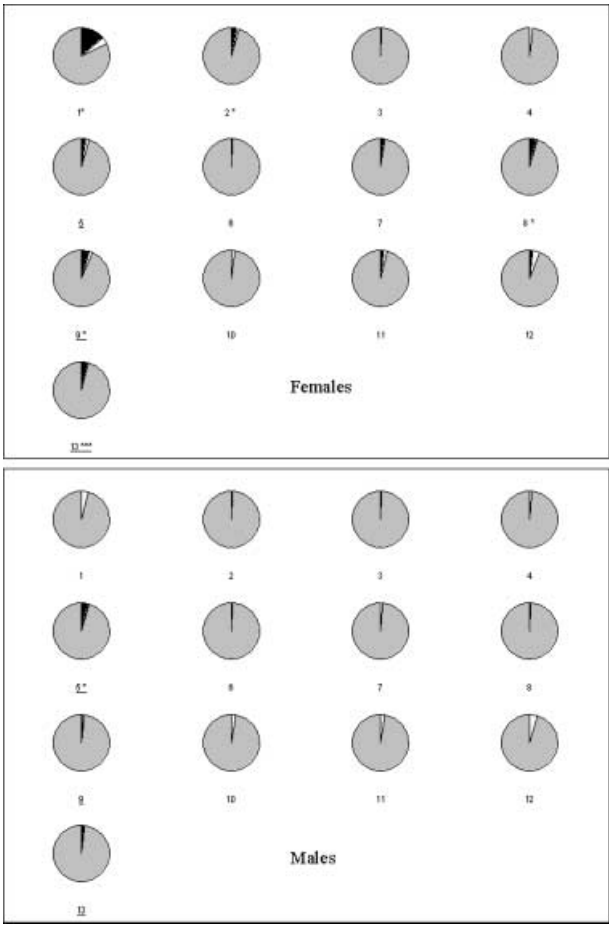

Fig. 3 Pie charts indicating the percentage of the variance for the effects of temperature (black), replicate (white) and error (grey) in the Procrustes ANOVAS on individual landmarks. Those landmarks used to estimate shape index (basal length/wing length) are underlined. Significance of temperature effect are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$).

at each landmark) does not seem to hold (see Dryden & Mardia, 1998, p. 97). The effects of between-individual variation were distributed in a relatively even way among all landmarks. Outer landmarks tended to move in the direction of an enlarged wing aspect; namely, to decrease wing width relative to WL. However, the direction of PCs is arbitrary and all the movements can be simultaneously reversed by 180°. Therefore, the best interpretation is to describe overall shape variation along a wide–narrow direction. The PC2 mainly consisted of a widening (narrowing) of the posterior compartment of the wing (which includes those landmarks located below an imaginary line situated approximately along the fourth longitudinal vein; see García-Bellido & de Celis, 1992). The PC3 simultaneously involved two landmarks on the fourth longitudinal vein (5 and 13) shifting to reverse directions, resulting in an increase (decrease) of the length of the distal segment L2. Conventional morphometric methods performed on wing size measurements in samples from Europe and North America also detected an inverse relationship (accounting for 10% of the variance) between the proximal (L1) and distal (L2) portions of longitudinal vein IV, as well as between WL and width (Gilchrist *et al.*, 2001).

For temperature effects there are only two PCs, and the features of shape variation are graphically shown in Fig. 5. Some slight differences between sexes are now apparent (but recall that Procrustes ANOVAS did not detect statistically significant thermal effects on males; Table 3). The PC1 in females was connected to the large variability previously detected for landmark 1 (see Fig. 3), which moves towards landmark 9. However, several landmarks had relatively large PC1 coefficients in males. Landmarks 13 and 7, which define the position of the posterior cross vein, shift to the same direction in both sexes. Similar to PC3s for individual variation, landmarks 5 and 13 seem to move in opposite directions but these shifts were quite small in females. The PC2 was mainly involved with the shift of landmark 5 in males and females.

Rates of genetic divergence for wing shape

Rates of wing size evolution and divergence on a continental scale have been estimated to be very fast in *D. subobscura* (Huey *et al.*, 2000; Gilchrist *et al.*, 2001). We did not detect here any significant effect of thermal selection regimes on wing size, but it could be interesting to estimate the rates of divergence for wing shape in the experimental populations (Hendry & Kinnison, 1999 describe this as the synchronic method). To handle wing shape as a single metric we used Procrustes distances and calculated them as $\rho = 2 \sin^{-1}(d_p/2)$, where d_p is the square root of the sum of square differences between corresponding points (see Rohlf, 1999). This procedure obviously reduces the inherently multidimensional shape data to a single magnitude of shape differences,

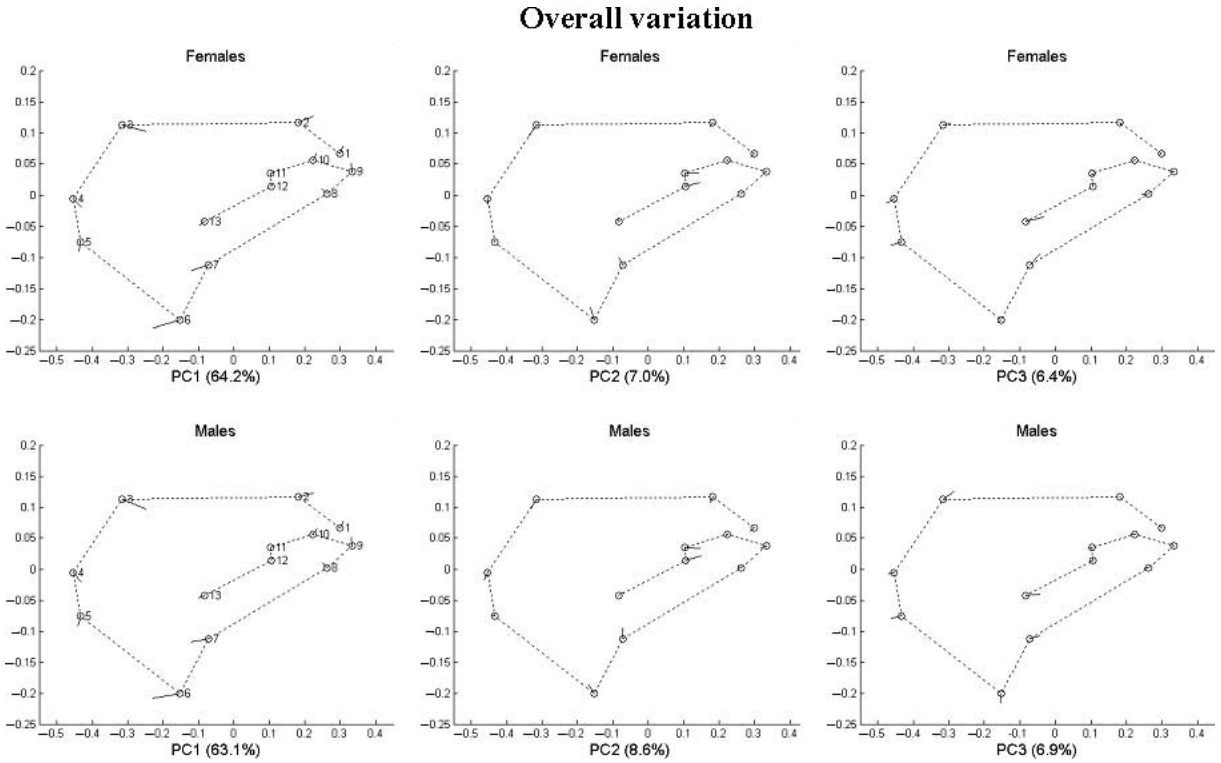

Fig. 4 Principal components of the covariance patterns in landmarks shifts due to among-individual variation for each sex. The PC coefficients are shown as a solid line originating at the mean location of the landmark (open circles) and ending at the location to which the landmark would move to +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations (obviously an exaggeration of the variation in the dataset). The proportion of total variation accounted by each PC is given in brackets.

and ignores the direction of landmarks' shifts against each other (Goodall, 1991; Klingenberg, 2003; but see Monteiro *et al.*, 2003). However, the Procrustes distances are informative as summary statistics and can be used to investigate evolutionary rates of shape change.

Two-level nested ANOVAS applied to the Procrustes distances also rendered significant and consistent (across replicated populations) shape variation among thermal selection regimes for both sexes (females: $F_{44,132} = 6.97$, $P < 0.05$; males: $F_{44,132} = 7.51$, $P < 0.05$. Probabilities were computed after 10 000 random permutations as described in Materials and methods); and *post hoc* comparisons detected statistically significant differences when contrasting 18 °C vs. 13 °C or 22 °C in females, and 13 °C vs. 22 °C in males (analyses not shown). Incidentally, although qualitative conclusions obtained from shape index L1/WL (see Table 2 and above) and from Procrustes distances are the same, both variables are loosely correlated (females: Spearman $r_S = -0.020$, n.s.; males: $r_S = -0.068$, $P < 0.05$).

Divergence rates for wing shape were estimated to be 0.0105 haldanes (2.1×10^4 darwins; 13 °C vs. 18 °C) and 0.0106 haldanes (2.7×10^4 darwins; 18 °C vs. 22 °C) in females, and 0.0068 haldanes (1.7×10^4 darwins; 13 °C vs. 22 °C) in males. Our approach of comparing only those means that showed statistically significant differences in *post hoc* contrasts can be obviously criticized (see Hendry & Kinnison, 1999). However, the previous figures do suggest that rates of genetic divergence for wing shape can be as fast or even faster than those estimated for wing size (cf. with the values reported by Gilchrist *et al.*, 2001).

Relationship between chromosomal polymorphism and wing shape index

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 year of divergence are shown in Table 4. Three-way log-linear analyses (including experimental temperature, replicate, and gene arrangement as the main effects) performed for each chromosome showed that the frequencies of some gene arrangements have already changed according to experimental temperature, but the three replicate populations within each temperature were homogeneous and could be lumped together (results not shown). Our aim here, however, is not to discuss those changes in gene arrangement frequencies but to relate the simultaneously analysed chromosomal

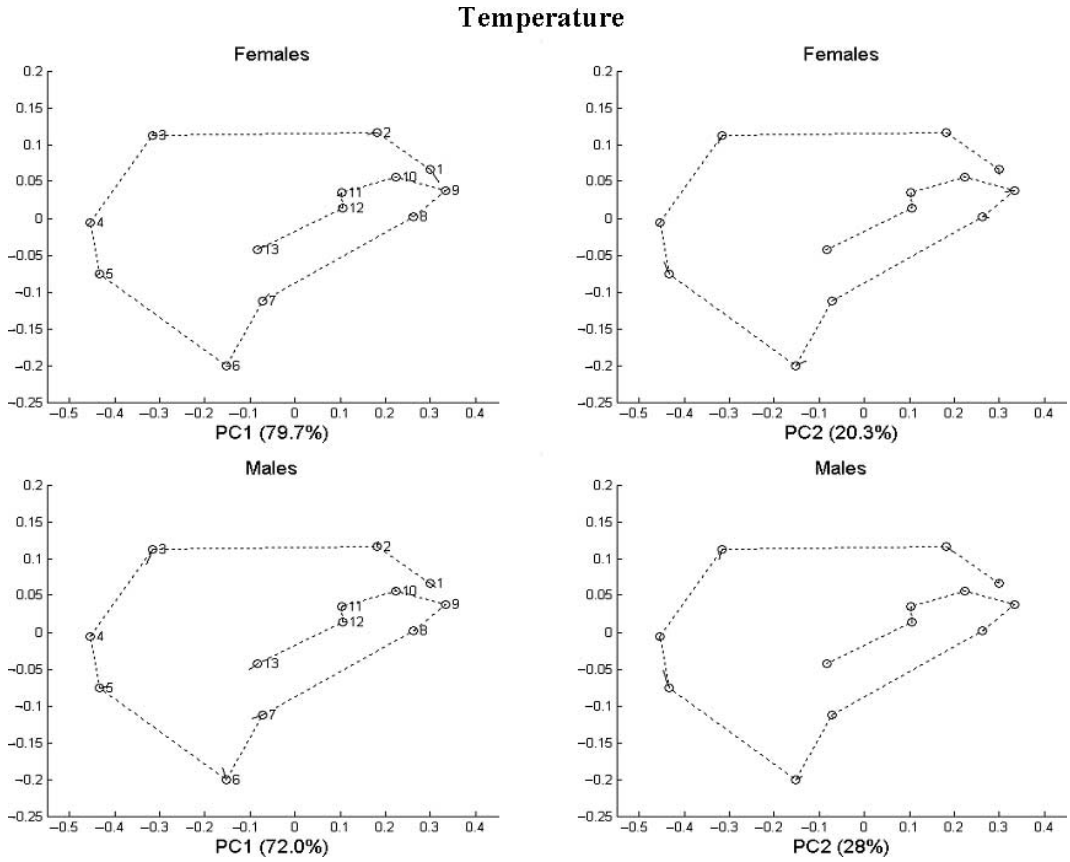

Fig. 5 Principal components of the covariance patterns in landmarks shifts due to among-temperature variation for each sex. The end points of the solid lines are at locations displaced +15 (PC1) and +30 (PC2) standard deviations from the mean configuration.

polymorphism and wing shape index in the males sampled from the thermal stocks. For this purpose we have used the 'standard dose' (i.e. the number of standard gene arrangements carried out by a male, which ranges from 0 to 5) as the relevant variable. The reason is that the various gene arrangements in Table 4 can be divided in two groups based on the correlation of gene arrangement frequencies and latitude: the 'cold-adapted' group comprised by A_{st}, J_{st}, U_{st}, E_{st} and O_{st}; and the 'warm-adapted' group involving A_2, J_1, U_{1+2}, U_{1+2+8}, $E_{1+2+9+3}$, $E_{1+2+9+12}$, O_{3+4} and O_{3+4+8} (arrangements E_{1+2+9}, O_{3+4+2} and O_{3+4+7} do not show a clear latitudinal pattern; see Menozzi & Krimbas, 1992). Because the frequencies of some standard gene arrangements were relatively low, we have grouped the low frequencies at the upper tail of the distribution and the relationship between shape index L1/WL and the standard dose is plotted in Fig. 6 for each experimental temperature. In all cases, the length of L1 relative to total WL sharply decreased with the standard dose [dependent variable $\log_e$(L1/WL); 13 °C: $\beta = -5.16 \times 10^{-3}$, $F_{1,328} = 20.11$, $P < 0.001$; 18 °C: $\beta = -3.85 \times 10^{-3}$, $F_{1,358} = 12.37$, $P < 0.001$; 22 °C: $\beta = -6.46 \times 10^{-3}$, $F_{1,324} = 26.23$, $P < 0.001$], and the regression coefficients were not statistically heterogeneous [$F_{2,1010} = 1.18$, n.s. Overall $\beta = -5.10 \times 10^{-3}$ (95% confidence limits: -6.42×10^{-3}, -3.78×10^{-3})]. It thus seems quite clear that polymorphic gene arrangements in *D. subobscura* have a consistent (across temperatures) biometrical effect on wing shape. The standard dose in the thermal stocks increased with increasing temperature, which could somewhat explain the pattern observed in Fig. 2 for males (in fact, the statistically significant effect for temperature in Table 2b disappears when the standard dose is introduced in the analysis as a covariate; results not shown). Interestingly, the decrease of wing shape index with latitude observed in North American colonizing populations (Huey *et al.*, 2000) fully agrees with the present data because the standard gene arrangements generally increase with latitude in those populations (Balanyà *et al.*, 2003). Actually, the increase of standard dose with latitude for eight North American populations covering a latitudinal range of about 13° and sampled in 1994 was $\beta = 0.072 \pm 0.010$. As formerly pointed out we did not aim here to discuss the changes in gene arrangement frequencies in the thermal selection stocks, but just

Table 4 Chromosomal polymorphism in the original natural population at Puerto Montt, in the founding population, and in the thermal selection stocks after 1 year.

			Experimental populations											
	Natural	Founding	13 °C (generation 9) (May 2002)				18 °C (generation 12) (May 2002)				22 °C (generation 15) (May 2002)			
Chromosome arrangement	population (November 1999)	population (May 2001)	R1	R2	R3	Total	R1	R2	R3	Total	R1	R2	R3	Total
A														
A_{st}	0.507	0.306	0.248	0.157	0.236	0.213	0.198	0.226	0.258	0.227	0.096	0.164	0.224	0.160
A_2	0.493	0.694	0.752	0.843	0.764	0.787	0.802	0.774	0.742	0.773	0.904	0.836	0.776	0.840
N	134	121	117	115	110	342	126	124	120	370	114	110	107	331
J														
J_{st}	0.304	0.281	0.145	0.118	0.089	0.118	0.108	0.114	0.194	0.138	0.190	0.188	0.189	0.189
J_1	0.696	0.719	0.855	0.882	0.911	0.882	0.892	0.886	0.806	0.862	0.810	0.813	0.811	0.811
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339
U														
U_{st}	0.422	0.512	0.265	0.269	0.277	0.270	0.312	0.220	0.239	0.257	0.448	0.339	0.351	0.381
U_{1+2}	0.348	0.281	0.538	0.479	0.482	0.500	0.529	0.591	0.515	0.545	0.353	0.438	0.477	0.422
U_{1+2+8}	0.230	0.207	0.197	0.252	0.241	0.230	0.159	0.189	0.246	0.198	0.198	0.223	0.171	0.198
N	135	121	117	119	112	348	138	132	134	404	116	112	111	339
E														
E_{st}	0.593	0.612	0.530	0.462	0.491	0.494	0.755	0.659	0.709	0.709	0.828	0.786	0.865	0.826
E_{1+2}	0.037	–	–	–	–	–	–	–	–	–	–	–	–	–
E_{1+2+9}	0.059	0.074	0.034	0.067	0.027	0.043	0.036	0.098	0.022	0.052	0.112	0.045	0.045	0.068
$E_{1+2+9+3}$	0.148	0.231	0.214	0.168	0.161	0.181	0.101	0.076	0.179	0.119	0.043	0.116	0.063	0.074
$E_{1+2+9+12}$	0.163	0.083	0.222	0.303	0.321	0.282	0.108	0.167	0.090	0.121	0.017	0.054	0.027	0.032
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339
O														
O_{st}	0.289	0.190	0.111	0.109	0.045	0.089	0.101	0.098	0.082	0.094	0.121	0.018	0.090	0.077
O_{3+4}	0.081	0.050	0.068	0.034	0.080	0.060	0.072	0.045	0.075	0.064	0.181	0.259	0.189	0.209
O_{3+4+2}	0.252	0.421	0.581	0.588	0.625	0.598	0.619	0.606	0.500	0.575	0.534	0.455	0.550	0.513
O_{3+4+7}	0.126	0.140	0.085	0.143	0.116	0.115	0.108	0.114	0.179	0.133	0.043	0.125	0.108	0.091
O_{3+4+8}	0.170	0.149	0.154	0.109	0.125	0.129	0.101	0.136	0.157	0.131	0.121	0.125	0.063	0.103
O_5	0.074	0.050	–	0.008	0.009	0.006	–	–	0.007	0.002	–	0.018	–	0.006
O_7	0.007	–	–	0.008	–	0.003	–	–	–	–	–	–	–	–
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339

notice that the trend between the standard dose and temperature in these stocks after 1 year of divergence goes in the opposite direction to that expected from the latitudinal clines.

Discussion

Flies of both sexes that have diverged for 27 generations at most were found to be about the same size independently of thermal selection regime. The lack of divergence in wing size could probably have been expected from results in the first surveys conducted on *D. subobscura* flies from New World populations, which failed to show any latitudinal size clines 8 years after colonization (~40 generations; see Pegueroles *et al.*, 1995). Further samples from the laboratory populations collected after longer divergence times will allow us to test predictions based on the size clines. However, features of wing shape that differ between experimental treatments (and sexes) were found to be consistent within the three replicated populations, which clearly excludes any explanation of wing shape variation in *D. subobscura* grounded on drift around an optimum (Gilchrist *et al.*, 2001; see below).

Earlier experiments in *Drosophila* had also detected progressive differences in wing form between (unreplicated) stocks maintained at different temperatures (Cavicchi *et al.*, 1978). The changes, however, were not significant in any single dimension but were detected after a multivariate analysis. Further artificial selection experiments (Cavicchi *et al.*, 1981) led the authors to the conclusion that the individual wing dimensions are largely inseparable genetically. However, Weber (1990) has rightly argued that a shortcoming with the latter experiment is that the authors only selected on one wing dimension and in one direction, which is equivalent to directional selection for size (but not shape) because most of the genetic variance for size in any single dimension is simply variance for total size. When artificial selection is performed on angular offsets extensive localized effects are revealed, making it clear that the wing does not

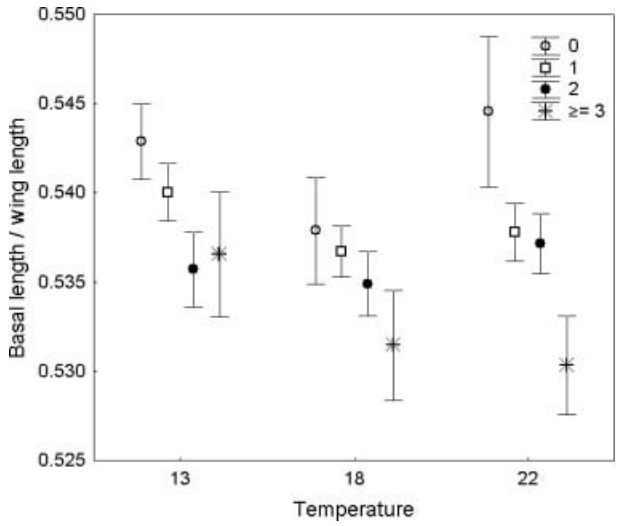

Fig. 6 Averages of the relative length (with 95% confidence intervals) of the basal portion of longitudinal vein IV (L1) to the total wing length (WL = L1 + L2) according to the dose (0, 1, 2 and ≥3) of standard gene arrangements carried by the sampled males at each experimental temperature. All three replicated populations within each thermal selection regime were pooled (see text for details).

evolve as a whole unit (Weber, 1990). Nevertheless, some contrasting patterns between overall wing variation (Fig. 4) and temperature effects (Figs 3 and 5) are apparent in our data set. In the first case the shifts of landmarks in PC1 did not occur in isolation, but also included most landmarks. Temperature has more localized effects in the shifts of landmarks. Similarly to the finding by Klingenberg & Zaklan (2000) in *D. melanogaster*, the shifts of the anterior (PC2 in Fig. 4) and posterior (PC3) cross veins appear to be rather independent of each other. These authors discuss similar displacements detected in both intra- and interspecific studies of wild-type flies, as well as in a number of mutant stocks.

In contrast to what has been uncovered for wing size, there are no consistent patterns between latitude and wing shape in *Drosophila*. Shape index L1/WL in *D. subobscura* increases with latitude in Europe and decreases in North America (Huey *et al.*, 2000), but shows no linear trend in South America (G. W. Gilchrist, pers. comm., 2002). In *D. melanogaster* Gilchrist *et al.* (2000) found that the main shape canonical variate also varied significantly between continents. Hoffmann & Shirriffs (2002) found a nonlinear trend between latitude and the first shape canonical variate in *D. serrata* flies from Australia, although there was a linear change in wing aspect (the ratio of WL to wing width). Parenthetically, the shape changes brought about by thermal selection regimes in our *D. subobscura* stocks were not associated with changes in wing-aspect ratio [estimated as (wing length)^2/wing area (see Azevedo *et al.*, 1998). Females: $F_{2,6} = 1.31$, n.s.; males: $F_{2,6} = 0.84$, n.s.]. Overall the results of these studies, as far as can be judged from the published reports on traditional and geometric morphometrics, quantitative genetics and QTL analyses of *Drosophila* wing shape (Weber, 1990, 1992; Bitner-Mathé & Klaczko, 1999a,b; Weber *et al.*, 1999, 2001; Birdsall *et al.*, 2000; Klingenberg & Zaklan, 2000; Zimmerman *et al.*, 2000; Gilchrist & Partridge, 2001), suggest that size and shape have different genetic properties and do not respond to the same environmental factors. In summary, the steady geographical clines for size across continents and *Drosophila* species, with wing size increasing with latitude largely independently of the underlying details in the genetic architecture (Gilchrist & Partridge, 1999), strongly support the hypothesis that body size (or the correlated trait growth rate) is the target of selection but there is a localized and richly structured variation in wing shape.

As wing shape seems to be strongly resistant to environmental influences (Weber, 1990; Birdsall *et al.*, 2000) and there is little compelling evidence indicating that natural wing shape changes are adaptive in *Drosophila*, it would be premature to attempt to explain the high rates of genetic divergence in our thermal selection stocks in functional terms [notwithstanding Imasheva *et al.*'s (1995) conclusions]. Wing shape is indeed remarkably constant within inbred lines (Birdsall *et al.*, 2000); however, it does not seem to be strongly canalized against genetic change and responds to divergent selection in the same way as most quantitative traits, with some genes causing small and localized effects (Weber, 1990, 1992). Many genes with small additive effects on wing shape are dispersed along the *Drosophila* genome (Weber *et al.*, 1999, 2001; Zimmerman *et al.*, 2000). This suggests plentiful chances for gene-inversion linkage disequilibria in the inversion-rich species *D. subobscura*, particularly in samples derived from New World colonizing populations (actually, linkage disequilibria between microsatellite loci are almost absent in European populations but are detectable in New World populations; M. Pascual, pers. comm., 2002). The thermal selection stocks have already diverged for various gene arrangements with no differences between replicated populations (see above), and a strong relationship between shape variables and polymorphic inversions (i.e. shape index L1/WL sharply decreased at all experimental temperatures as the dose of standard chromosomal arrangements increased) was uncovered. In addition, our findings strongly suggest that the latitudinal clines for chromosomal gene arrangements may account for the wing shape cline in North America colonizing populations (Huey *et al.*, 2000). Even more, we could hypothesize that the shape cline in North America predated the size cline because of the quite different paces between inversion and size clines (see above). To test this hypothesis we have reanalysed the data reported in Pegueroles *et al.* (1995) for six North American populations sampled by A. Prevosti and M. Monclús in July 1986 (4 years after the

initial colonization in North America) before the size cline built up. As predicted, for both females [dependent variable $\log_e$(L1/WL); $\beta = -0.92 \pm 0.27 \times 10^{-3}$] and males ($\beta = -0.49 \pm 0.26 \times 10^{-3}$) the length of L1 relative to total WL decreased with latitude, but the colonizing males were lagging behind females and this tendency is still apparent in more recent samples (see Gilchrist *et al.*, 2001). In summary, our results cast strong doubts on the supposed 'unpredictability' of the geographical cline in *D. subobscura* North American colonizing populations (Huey *et al.*, 2000).

The use of geometric morphometrics together with quantitative genetic studies is an ambitious project and requires large data sets (see Klingenberg & Leamy, 2001; Klingenberg, 2003). We are currently analysing male's multivariate wing shape tangent space in relation to inversions, in addition to carrying out quantitative genetic analyses in a set of isochromosomal lines fixed for different O chromosome arrangements. In the meantime, the most parsimonious explanation for our present results is that changes in gene arrangement frequencies as a response to temperature likely underlie the correlated changes in wing shape because of gene-inversion linkage disequilibria.

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References

Anderson, W.W. 1966. Genetic divergence in M. Vetukhiv's experimental populations of *Drosophila pseudoobscura*. *Genet. Res. Camb.* **7**: 255–266.

Azevedo, R.B.R., French, V. & Partridge, L. 1996. Thermal evolution of egg size in *Drosophila melanogaster*. *Evolution* **50**: 2338–2345.

Azevedo, R.B.R., James, A.C., McCabe, J. & Partridge, L. 1998. Latitudinal variation of wing: thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution* **52**: 1353–1362.

Balanyà, J., Serra, L., Gilchrist, G.W., Huey, R.B., Pascual, M., Mestres, F. & Solé, E. 2003. Evolutionary pace of chromosomal polymorphism in colonizing populations of *Drosophila subobscura*: an evolutionary time series. *Evolution* **57**: 1837–1845.

Beckenbach, A.T. & Prevosti, A. 1986. Colonisation of North America by the European species *Drosophila subobscura* and *Drosophila ambigua*. *Am. Midl. Nat.* **115**: 10–18.

Birdsall, K., Zimmerman, E., Teeter, K. & Gibson, G. 2000. Genetic variation for the positioning of wing veins in *Drosophila melanogaster*. *Evol. Devel.* **2**: 16–24.

Bitner-Mathé, B.C. & Klaczko, L.B. 1999a. Heritability, phenotypic and genetic correlations of size and shape of *Drosophila mediopunctata* wings. *Heredity* **83**: 688–696.

Bitner-Mathé, B.C. & Klaczko, L.B. 1999b. Size and shape heritability in natural populations of *Drosophila mediopunctata*: temporal and microgeographical variation. *Genetica* **105**: 35–42.

Bochdanovits, Z. & De Jong, G. 2003. Experimental evolution in *Drosophila melanogaster*: interaction of temperature and food quality selection regimes. *Evolution* **57**: 1829–1836.

Bookstein, F.L. 1991. *Morphometric Tools for Landmark Data: Geometry and Biology*. Cambridge University Press, Cambridge, UK.

Bookstein, F.L. 1996. Biometrics, biomathematics and the morphometric synthesis. *Bull. Math. Biol.* **58**: 313–365.

Borash, D.J., Gibbs, A.G., Joshi, A. & Mueller, L.D. 1998. A genetic polymorphism maintained by natural selection in a temporally varying environment. *Am. Nat.* **151**: 148–156.

Borash, D.J., Pierce, V.A., Gibbs, A.G. & Mueller, L.D. 2000. Evolution of ammonia and urea tolerance in *Drosophila melanogaster*: resistance and cross-tolerance. *J. Insect Physiol.* **46**: 763–769.

Brncic, D., Prevosti, A., Budnik, M., Monclús, M. & Ocaña, J. 1981. Colonization of *Drosophila subobscura* in Chile. I. First population and cytogenetics studies. *Genetica* **56**: 3–9.

Calboli, F.C.F., Gilchrist, G.W. & Partridge, L. 2003. Different cell size and cell number contribution in two newly established and one ancient body size cline of *Drosophila subobscura*. *Evolution* **57**: 566–573.

Cavicchi, S., Giorgi, D. & Mochi, M. 1978. Investigation of early divergence between populations of *Drosophila melanogaster* kept at different temperatures. *Genetica* **48**: 81–87.

Cavicchi, S., Pezzoli, C. & Giorgi, G. 1981. Correlation between characters as related to developmental pattern in *Drosophila*. *Genetica* **56**: 189–195.

Cavicchi, S., Guerra, D., Giorgi, G. & Pezzoli, C. 1985. Temperature-related divergence in experimental populations of *Drosophila melanogaster*. I. Genetic and developmental basis of wing size and shape variation. *Genetics* **109**: 665–689.

Cavicchi, S., Guerra, D., Natali, V., Pezzoli, C. & Giorgi, G. 1989. Temperature-related divergence in experimental populations of *Drosophila melanogaster*. II. Correlation between fitness and body dimensions. *J. Evol. Biol.* **2**: 235–251.

Chapman, R.E. 1990. Conventional Procrustes approaches. In: *Proceedings of the Michigan Morphometrics Workshop* (F. J. Rohlf & F. L. Bookstein, eds), pp. 251–267. Special Publication No. 2. University of Michigan Museum of Zoology, Ann Arbor, MI.

Coyne, J.A. & Beecham, E. 1987. Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. *Genetics* **117**: 727–737.

Crill, W.D., Huey, R.B. & Gilchrist, G.W. 1996. Within- and between-generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*. *Evolution* **50**: 1205–1218.

David, J. 1962. A new medium for rearing *Drosophila* in axenic conditions. *Dros. Inf. Serv.* **36**: 128.

David, J., Bocquet, C. & De Scheemaeker-Louis, M. 1977. Genetic latitudinal adaptation of *Drosophila melanogaster*: new discriminative biometrical traits between European and equatorial African populations. *Genet. Res. Camb.* **30**: 247–255.

Dryden, I.L. & Mardia, K.V. 1998. *Statistical Shape Analysis*. John Wiley & Sons, Chichester.

García-Bellido, A. & de Celis, J.F. 1992. Developmental genetics of the venation pattern of *Drosophila*. *Annu. Rev. Genet.* **26**: 275–304.

Gilchrist, A.S. & Partridge, L. 1999. A comparison of the genetic basis of wing size divergence in three parallel body size clines of *Drosophila melanogaster*. *Genetics* **153**: 1775–1787.

Gilchrist, A.S. & Partridge, L. 2001. The contrasting genetic architecture of wing size and shape in *Drosophila melanogaster*. *Heredity* **86**: 144–152.

Gilchrist, A.S., Azevedo, R.B.R., Partridge, L. & O'Higgins, P. 2000. Adaptation and constrain in the evolution of *Drosophila melanogaster* wing shape. *Evol. Devel.* **2**: 114–124.

Gilchrist, G.W., Huey, R.B. & Serra, L. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. *Genetica* **112–113**: 273–286.

Goodall, C. 1991. Procrustes methods in the statistical analysis of shape. *J. R. Stat. Soc. B* **53**: 285–339.

Guo, P.Z., Mueller, L.D. & Ayala, F.J. 1991. Evolution of behavior by density-dependent selection. *Proc. Natl. Acad. Sci. USA* **88**: 10905–10906.

Hendry, A. & Kinnison, M.T. 1999. Perspective: the pace of modern life: measuring rates of contemporary microevolution. *Evolution* **53**: 1637–1653.

Hoffmann, A.A. & Shirriffs, J. 2002. Geographic variation for wing shape in *Drosophila serrata*. *Evolution* **56**: 1068–1073.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, P. J., Dai, X., Maskell, K. & Johnson, C. A. (eds) 2001. *Climate Change 2001: The Scientific Basis: Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, New York.

Houle, D. & Rowe, L. 2003. Natural selection in a bottle. *Am. Nat.* **161**: 50–67.

Huey, R.B., Partridge, L. & Fowler, K. 1991. Thermal sensitivity of *Drosophila melanogaster* responds rapidly to laboratory natural selection. *Evolution* **45**: 751–756.

Huey, R.B., Gilchrist, G.W., Carlson, M.L., Berrigan, D. & Serra, L. 2000. Rapid evolution of a geographical cline in size in an introduced fly. *Science* **287**: 308–309.

Imasheva, A.G., Bubli, O.A., Lazebny, O.E. & Zhivotovsky, L.A. 1995. Geographic differentiation in wing shape in *Drosophila melanogaster*. *Genetica* **96**: 303–306.

James, A.C. & Partridge, L. 1995. Thermal evolution of rate of larval development in *Drosophila melanogaster* in laboratory and field populations. *J. Evol. Biol.* **8**: 315–330.

James, A.C., Azevedo, R.B.R. & Partridge, L. 1997. Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. *Genetics* **146**: 881–890.

Jolliffe, I.T. 1986. *Principal Component Analysis*. Springer-Verlag, New York.

Joshi, A. & Mueller, L.D. 1988. Evolution of higher feeding rate in *Drosophila* due to density-dependent natural selection. *Evolution* **42**: 1090–1093.

Joshi, A. & Mueller, L.D. 1993. Directional and stabilizing density-dependent natural selection for pupation height in *Drosophila melanogaster*. *Evolution* **47**: 176–184.

Joshi, A. & Mueller, L.D. 1996. Density-dependent natural selection in *Drosophila*: trade-offs between larval food acquisition and utilization. *Evol. Ecol.* **10**: 463–474.

Klingenberg, C.P. 2003. Quantitative genetics of geometric shape: heritability and the pitfalls of the univariate approach. *Evolution* **57**: 191–195.

Klingenberg, C.P. & Leamy, L.J. 2001. Quantitative genetics of geometric shape in the mouse mandible. *Evolution* **55**: 2342–2352.

Klingenberg, C.P. & McIntyre, G.S. 1998. Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with Procrustes methods. *Evolution* **52**: 1363–1375.

Klingenberg, C.P. & Zaklan, S.D. 2000. Morphological integration between developmental compartments in the *Drosophila* wing. *Evolution* **54**: 1273–1285.

Koske, Th. & Maynard Smith, J. 1954. Genetics and cytology of *Drosophila subobscura*. X. The fifth linkage group. *J. Genet.* **52**: 521–541.

Krimbas, C.B. 1993. *Drosophila subobscura*. Biology, Genetics and Inversion Polymorphism. Verlag Dr Kovač, Hamburg.

Krimbas, C.B. & Loukas, M. 1980. The inversion polymorphism of *Drosophila subobscura*. *Evol. Biol.* **12**: 163–234.

van't Land, J., van Putten, P., Zwaan, B., Kamping, A. & van Delden, W. 1999. Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities and reaction norms. *J. Evol. Biol.* **12**: 222–232.

Lankinen, P. & Pinsker, W. 1977. Allozyme constitution of standard strains of *Drosophila subobscura*. *Experientia* **33**: 1301.

Manly, B.F.J. 1997. *Randomization, Bootstrap and Monte Carlo Methods in Biology*, 2nd edn. Chapman & Hall, London.

Matos, M., Rose, M.R., Rocha Pité, M.T., Rego, C. & Avelar, T. 2000. Adaptation to the laboratory environment in *Drosophila subobscura*. *J. Evol. Biol.* **13**: 9–19.

Mayr, E. 1942. *Systematics and the Origin of Species*. Columbia University Press, New York.

Menozzi, P. & Krimbas, C.B. 1992. The inversion polymorphism of *Drosophila subobscura* revisited: synthetic maps of gene arrangement frequencies and their interpretation. *J. Evol. Biol.* **5**: 625–641.

Mestres, F., Balañá, J., Segarra, C., Prevosti, A. & Serra, L. 1992. Colonization of America by *Drosophila subobscura*: analysis of the O_5 inversions from Europe and America and their implications for the colonizing process. *Evolution* **46**: 1564–1568.

Misra, R.K. & Reeve, E.C.R. 1964. Clines in body dimensions in populations of *Drosophila subobscura*. *Genet. Res. Camb.* **5**: 240–256.

Monteiro, L.R., Diniz-Filho, J.A.F., dos Reis, S.F. & Araujo, E.D. 2003. Shape distances in general linear models: are they really at odds with the goals of morphometrics? A reply to Klingenberg. *Evolution* **57**: 196–199.

Mousseau, T.A. 1997. Ectotherms follow the converse to Bergmann's rule. *Evolution* **51**: 630–632.

Mueller, L.D., Graves, J.L. & Rose, M.R. 1993. Interactions between density-dependent and age-specific selection in *Drosophila melanogaster*. *Funct. Ecol.* **7**: 469–479.

Needham, J. 1950. *Biochemistry and Morphogenesis*, 2nd edn. Cambridge University Press, Cambridge.

Orengo, D.J. & Prevosti, A. 1996. Temporal changes in chromosomal polymorphism of *Drosophila subobscura* related to climatic changes. *Evolution* **50**: 1346–1350.

Orengo, D.J. & Prevosti, A. 2002. Relationship between chromosomal polymorphism and wing size in a natural population of *Drosophila subobscura*. *Genetica* **115**: 311–318.

Partridge, L. & Coyne, J.A. 1997. Bergmann's rule in ectotherms: is it adaptive? *Evolution* **51**: 632–635.

Partridge, L., Barrie, B., Fowler, K. & French, V. 1994a. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. *Evolution* **48**: 1269–1276.

Partridge, L., Barrie, B., Fowler, K. & French, V. 1994b. Thermal evolution of pre-adult life-history traits in *Drosophila melanogaster*. *J. Evol. Biol.* **7**: 645–663.

Pegueroles, G., Papaceit, M., Quintana, A., Guillén, A., Prevosti, A. & Serra, L. 1995. An experimental study of evolution in progress: clines for quantitative traits in colonizing and Paleartic populations of *Drosophila*. *Evol. Ecol.* **9**: 453–465.

Pfriem, P. 1983. Latitudinal variation in wing size in *Drosophila subobscura* and its dependence on polygenes of chromosome O. *Genetica* **61**: 221–232.

Powell, J.R. 1974. Temperature related divergence in *Drosophila* body size. *J. Hered.* **65**: 257–258.

Prevosti, A. 1955. Geographical variability in quantitative traits in populations of *Drosophila subobscura*. *Cold Spring Harbor Symp. Quant. Biol.* **20**: 294–298.

Prevosti, A., Serra, L., Ribó, G., Aguadé, M., Sagarra, E., Monclús, M. & García, M.P. 1985. The colonization of *Drosophila subobscura* in Chile. II. Clines in the chromosomal arrangements. *Evolution* **39**: 838–844.

Prevosti, A., Ribó, G., Serra, L., Aguadé, M., Balañá, J., Monclús, M. & Mestres, F. 1988. Colonization of America by *Drosophila subobscura*: experiment in natural populations that supports the adaptive role of chromosomal-inversion polymorphism. *Proc. Natl. Acad. Sci. USA* **85**: 5597–5600.

Robertson, F.W. & Reeve, E.C.R. 1952. Studies in quantitative inheritance. I. The effect of selection of wing and thorax length in *Drosophila melanogaster*. *J. Genet.* **50**: 414–448.

Rodríguez-Trelles, F. & Rodríguez, M.A. 1998. Rapid micro-evolution and loss of chromosomal diversity in *Drosophila* in response to climate warming. *Evol. Ecol.* **12**: 829–838.

Rodríguez-Trelles, F., Alvarez, G. & Zapata, C. 1996. Time-series analysis of seasonal changes of the O inversion polymorphism of *Drosophila subobscura*. *Genetics* **142**: 179–187.

Rohlf, F.J. 1990. Rotational fit (Procrustes) methods. In: *Proceedings of the Michigan Morphometrics Workshop* (F. J. Rohlf & F. L. Bookstein, eds), pp. 227–236. Special Publication No. 2. University of Michigan Museum of Zoology, Ann Arbor, MI.

Rohlf, F.J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. *J. Classif.* **16**: 197–223.

Rohlf, F.J. 2000. Statistical power comparisons among alternative morphometric methods. *Am. J. Phys. Anthropol.* **111**: 463–478.

Rohlf, F.J. 2003. Bias and error in estimates of mean shape in morphometrics. *J. Hum. Evol.* **44**: 665–683.

Rohlf, F.J. & Slice, D. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Syst. Zool.* **39**: 40–59.

Roper, C., Pignatelli, P. & Partridge, L. 1996. Evolutionary responses of *Drosophila melanogaster* life history to differences in larval density. *J. Evol. Biol.* **9**: 609–622.

Santos, M. 1996. Apparent directional selection of body size in *Drosophila buzzatii*: larval crowding and male mating success. *Evolution* **50**: 2530–2535.

Santos, M., Borash, D.J., Joshi, A., Bounlutay, N. & Mueller, L.D. 1997. Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. *Evolution* **51**: 420–432.

Slice, D.E., Bookstein, F.L., Marcus, L.F. & Rohlf, F.J. 1996. A glossary for geometric morphometrics. In: *Advances in Morphometrics* (L. F. Marcus, M. Corti, A. Loy, G. J. P. Naylor & D. E. Slice, eds), pp. 531–551. Plenum Press, New York (URL: http://life.bio.sunysb.edu/morph).

Sokal, R.R. & Rohlf, F.J. 1981. *Biometry*, 2nd edn. Freeman, New York.

Solé, E., Balanyà, J., Sperlich, D. & Serra, L. 2002. Long-term changes in the chromosomal inversion polymorphism of *Drosophila subobscura*. I. Mediterranean populations from southwestern Europe. *Evolution* **56**: 830–835.

SPSS Inc. 2001. *SPSS for Windows* (URL: http://www.spss.com).

Stalker, H.D. & Carson, H.L. 1947. Morphological variation in natural populations of *Drosophila robusta* Sturtevant. *Evolution* **1**: 237–248.

StatSoft, Inc. 2003. *STATISTICA (Data Analysis Software System)*, version 6. (URL: http://www.statsoft.com).

Stevenson, R.D. 1985. Body size and limits to daily range of body temperature in terrestrial ectotherms. *Am. Nat.* **125**: 102–117.

The MathWorks, Inc. 1998, 2002. *Matlab, V.5 and V.6. The Language of Technical Computing* (URL: http://www.mathworks.com).

Van Voorhies, W.A. 1997. On the adaptive nature of Bergmann size clines: a replay to Mousseau, Partridge and Coyne. *Evolution* **51**: 635–640.

Walker, J.A. 2000. Ability of geometric morphometric methods to estimate a known covariance matrix. *Syst. Biol.* **49**: 686–696.

Weber, K.E. 1990. Artificial selection on wing allometry in *Drosophila melanogaster*. *Genetics* **126**: 975–989.

Weber, K.E. 1992. How small are the smallest selectable domains of form? *Genetics* **130**: 345–353.

Weber, K., Eisman, R., Morey, L., Patty, A., Sparks, J., Tausek, M. & Zeng, Z.-B. 1999. An analysis of polygenes affecting wing shape on chromosome 3 in *Drosophila melanogaster*. *Genetics* **153**: 773–786.

Weber, K., Eisman, R., Higgins, S., Morey, L., Patty, A., Tausek, M. & Zeng, Z.-B. 2001. An analysis of polygenes affecting wing shape on chromosome 2 in *Drosophila melanogaster*. *Genetics* **159**: 1045–1057.

Zimmerman, E., Palsson, A. & Gibson, G. 2000. Quantitative trait loci affecting components of wing shape in *Drosophila melanogaster*. *Genetics* **155**: 671–683.

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