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Thermal evolution of pre-adult life history traits, geometric size and shape, and developmental stability in *Drosophila subobscura*

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Abstract

Replicated lines of *Drosophila subobscura* originating from a large outbred stock collected at the estimated Chilean epicentre (Puerto Montt) of the original New World invasion were allowed to evolve under controlled conditions of larval crowding for 3.5 years at three temperature levels (13, 18 and 22 °C). Several pre-adult life history traits (development time, survival and competitive ability), adult life history related traits (wing size, wing shape and wing-aspect ratio), and wing size and shape asymmetries were measured at the three temperatures. Cold-adapted (13 °C) populations evolved longer development times and showed lower survival at the highest developmental temperature. No divergence for wing size was detected following adaptation to temperature extremes (13 and 22 °C), in agreement with earlier observations, but wing shape changes were obvious as a result of both thermal adaptation and development at different temperatures. However, the evolutionary trends observed for the wing-aspect ratio were inconsistent with an adaptive hypothesis. There was some indication that wing shape asymmetry has evolutionarily increased in warm-adapted populations, which suggests that there is additive genetic variation for fluctuating asymmetry and that it can evolve under rapid environmental changes caused by thermal stress. Overall, our results cast strong doubts on the hypothesis that body size itself is the target of selection, and suggest that pre-adult life history traits are more closely related to thermal adaptation.

Introduction

Small invertebrates with short development times and several generations per year tend to show body size clines with genetically larger individuals derived from higher latitudes (reviewed in Blanckenhorn & Demont, 2004). For evolutionary biologists the occurrence of repeated patterns of clinal genetic variation in different continents, which in a well-documented case are known to have evolved within 20 years (*Drosophila subobscura*: Huey *et al.*, 2000; Gilchrist *et al.*, 2001, 2004; Calboli *et al.*, 2003), provides *prima facie* evidence of the power and predictability of natural selection over large geographic scales. Although there is agreement that such clines are probably caused by temperature, the snag is, however, that a compelling adaptive explanation still remains elusive as it is not clear what the principal targets of selection are.

In *Drosophila*, a plethora of studies at the intraspecific level have used quantitative traits to investigate climatic adaptation by comparing flies' performance in laboratory assays once samples from the field were raised under uniform conditions to remove environmental effects; e.g. body size (Coyne & Beecham, 1987; James *et al.*, 1997; Karan *et al.*, 1998b; Huey *et al.*, 2000), development time (James *et al.*, 1995; Norry *et al.*, 2001; Sgró & Blows, 2003; Griffiths *et al.*, 2005), developmental stability (Jenkins & Hoffmann, 2000), heat-induced male sterility (Rohmer *et al.*, 2004; David *et al.*, 2005), larval growth

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efficiency (Robinson & Partridge, 2001), larval survival and pre-adult competitive ability (James & Partridge, 1998), stress resistance (Krebs & Loeschcke, 1995a, b; Karan *et al.*, 1998a; Bubliy *et al.*, 2002; Hoffmann *et al.*, 2002, 2005), wing loading and wing-aspect ratio (Azevedo *et al.*, 1998; Norry *et al.*, 2001; Gilchrist & Huey, 2004), wing shape (Imasheva *et al.*, 1995; Bitner-Mathe & Klaczko, 1999; Gilchrist *et al.*, 2000; Gilchrist & Partridge, 2001; Hoffmann & Shirriffs, 2002; Griffiths *et al.*, 2005). For various traits latitudinal/altitudinal clines are clearly detected and can even persist for many generations under laboratory rearing conditions (Gockel *et al.*, 2001). However, except for body size many patterns are not usually consistent across studies or species. For instance, a weak clinal pattern for starvation resistance was detected in *D. melanogaster* populations from Australia (Hoffmann *et al.*, 2001) but not from South America (Robinson *et al.*, 2000). On the other hand, *D. melanogaster* populations from higher latitudinal regions in Australia had been reported to have a faster development (James & Partridge, 1995), but no clinal pattern was detected in a later survey (James *et al.*, 1997; see also Worthen, 1996). To further complicate things *D. birchii* (Griffiths *et al.*, 2005) and *D. serrata* (Sgró & Blows, 2003) show a contrasting cline with development time increasing from tropical to more temperate areas, but the trends in the latter species are clearly nonlinear, suggesting that selective factors other than those correlated with latitude are involved (Magiafoglou *et al.*, 2002). Additionally, in *D. melanogaster* (Gilchrist *et al.*, 2000) and *D. subobscura* (Huey *et al.*, 2000) wing shape features show contrasting latitudinal patterns between continents, but in *D. serrata* (Hoffmann & Shirriffs, 2002) and *D. birchii* (Griffiths *et al.*, 2005) from Australia wing shape variables did not show any linear trend with latitude. In *D. serrata*, however, there was a linear change in wing aspect (the ratio of wing length to wing width) that parallels that found in *D. melanogaster* geographic lines from the same continent (Azevedo *et al.*, 1998); namely, an increase in wing aspect with increasing latitude. Finally, Norry *et al.* (2001) go so far as to conclude that thermal adaptation in *D. buzzatii* is clear for pre-adult traits but not for size-related traits.

A wealth of supplementary information has also been amassed from *Drosophila* populations that have evolved at different temperatures in the laboratory (Anderson, 1966, 1973; Powell, 1974; Cavicchi *et al.*, 1985, 1989; Huey *et al.*, 1991; Partridge *et al.*, 1994a, b, 1995; Neat *et al.*, 1995; Gilchrist *et al.*, 1997; Bochdanovits & de Jong, 2003; Santos *et al.*, 2004, 2005a). In some cases the suit of traits that were affected by laboratory thermal evolution show patterns that are consistent with those predicted from latitudinal clines, clearly suggesting that temperature is the selective agent involved. However, while laboratory natural selection experiments with extensively replicated evolving populations are clearly a valuable research tool for understanding naturally occurring patterns, a critical problem with the so-called 'research program on experimental evolution' using laboratory-maintained populations (see http://irpee.bio.uci.edu/) is that unintended selection because of failures or difficulties in controlling potentially important factors (i.e. common elements of the culture regimes of different treatments that should be standardized) can seriously undermine the interpretation of results (Rose *et al.*, 1996; Harshman & Hoffmann, 2000).

We have previously discussed how larval culture density, a commonly neglected variable in laboratory thermal selection experiments, covaries with experimental temperature (e.g. *D. melanogaster* populations stabilize at lower densities when maintained at lower temperatures) and larval adaptation to different levels of crowding conditions which, in turn, may significantly impinge on other phases of the life cycle (Santos *et al.*, 2004, 2005a; see also Bochdanovits & de Jong, 2003; Prasad & Joshi, 2003, pp. 57, 58). Plainly said, a range of evolutionary responses to laboratory natural selection at different temperatures under uncontrolled larval densities (e.g. divergence in development time, pre-adult competitive ability, larval growth efficiency, size-dependent male mating success, starvation resistance, energy storage) could have been anticipated as a result of unintended correlated responses to various levels of larval crowding (Mueller, 1988; Joshi & Mueller, 1996; Santos, 1996; Santos *et al.*, 1997; Borash & Ho, 2001).

The main aim of this study was to investigate and reassess how temperature *per se* shapes a range of pre-adult and adult life history-related traits that have previously been claimed to respond to laboratory thermal selection. To be more specific, our expectation here is that the previously observed divergence in pre-adult life history traits (e.g. sensitivity to larval crowding, larval survival and larval growth efficiency), apparently as a result of thermal adaptation (Huey *et al.*, 1991; Partridge *et al.*, 1994b; James & Partridge, 1995), would likely have been different or have even vanished if the selective effects of temperature and resource availability (i.e. larval crowding) were experimentally disentangled. Bochdanovits & de Jong (2003) have analysed the interaction between temperature and food quality regimes by setting four experimental evolution lines of *D. melanogaster* at four different combinations, and found that thermal evolution of body size interact with the food quality regime. The lines were, however, kept with a relatively low number of breeding adults and were not replicated, so it seems highly desirable to complement their experiments.

We used three replicated populations of the temperate (i.e. cold tolerant) species *D. subobscura* kept at three thermal selection regimes on a discrete generation, regulated moderate larval densities (see Santos *et al.*, 2004): cold (13 °C), optimum (18 °C) and warm (22 °C). These populations had already diverged for wing shape, chromosomal diversity and gene arrangement frequencies

after 2 years of thermal selection, but not for wing size at the lowest and highest thermal regimes when tested at the rearing temperature of 18 °C (Santos *et al.*, 2004, 2005a). For the present assays the populations were sampled after 3.5 years. In addition to examining pre-adult life history traits (development time, viability and competitive ability), all populations were scored again for wing size and shape at the three developmental temperatures (13, 18 and 22 °C) to also investigate the phenotypic plasticity of these traits. Furthermore, trends in wing asymmetry were also looked at to test whether or not fluctuating asymmetry (FA, small random deviations from symmetry in otherwise bilaterally symmetrical characters; Van Valen, 1962; Palmer & Strobeck, 1986) evolves as a result of thermal selection. If so, this would imply that there is some amount of additive genetic variation in developmental stability that is usually difficult to detect when measured as heritability (Leamy & Klingenberg, 2005).

Materials and methods

Experimental populations and fly handling

The thermal selection populations have been described elsewhere (Santos *et al.*, 2004, 2005a). A key point to remember is that the large outbred base stock used to initiate those populations was collected at the estimated Chilean epicentre of the original New World invasion (Puerto Montt, Chile, 41°28′S, 73°00′W) All measurements in the present study were made after 3.5 years of thermal evolution (29 generations at 13 °C, 40 at 18 °C and 52 at 22 °C).

Before the experimental assays described below were performed, all test populations were passed through two generations of identical rearing conditions to eliminate any nongenetic parental thermal environment effects. Batches of 12 bottles from every thermal selection population (P13R1, P13R2, …, P22R3) were cultured at each of the three experimental temperatures (developmental temperature DT13, DT18 and DT22), and emerging adults were dumped into Plexiglas cages for egg collections. Eggs for the experiments were collected by placing Petri dishes containing nonnutritive agar with a generous smear of live yeast in the cages during 6 h at DT13, 2.5 h at DT18 and 2 h at DT22. All fly handling was done at room temperature using CO_2 anaesthesia when necessary on flies not less than 12 h after eclosion.

Pre-adult life history traits under uncrowded larval conditions

There was no indication of differences in embryonic development among the thermal selection regimes as judged by the hatching times of ~50% larvae from an initial sample of ~120 eggs (± 1 h) per population (the number of cast-off shells was checked microscopically every 15 min after the first larva was detected). The times from egg-lay to larval hatching were ~76 h at 13 °C, ~52 h at 18 °C and ~27 h at 22 °C.

Egg to adult viability and egg-lay to adult development time were measured at the three developmental temperatures under uncrowded larval conditions, resulting in a $3 \times 3 \times 3$ factorial design with thermal selection regime and developmental temperature as main fixed effects, and replicated populations as a random factor nested in thermal selection regime. For each thermal selection population and developmental temperature combination, eggs were placed in fifteen 2×8 cm vials (30 eggs/vial) with 6 mL of David's killed-yeast *Drosophila* medium (David, 1962). Within each developmental temperature all eggs were sampled on the same day and the vials were randomly placed on the same incubator shelf. Eclosing adults were collected every 8 h (08:00, 16:00 and 24:00), sexed after >12 h upon eclosion, and stored in Eppendorf tubes with a 3:1 mixture of alcohol and glycerol at 4 °C. Vials were scored until no new adults emerged for up to 72 h. The 12:12 light: dark period in the incubators was set from 07:00 (light) to 19:00 (dark). This is relevant here since eclosion peaks in *D. subobscura* occur early in the morning (very few flies were collected at 24:00).

Wing measurements

Two randomly sampled females and males that emerged from each of the uncrowded vials were used for morphometric analyses. Both wings were removed from each fly and fixed in DPX under coverslips on microscope slides. Images of left and right wings were captured as described in Santos *et al.* (2004). The *x* and *y* coordinates of 13 morphological landmarks (for an open access figure showing the landmarks used in this work see Santos *et al.*, 2005b, http://www.biomedcentral.com/1471-2148/5/7) were recorded by using the Fly Wing 15Lmk plug-in (kindly provided by Chris P. Klingenberg) implemented in ImageJ 1.33u software (http://rsb.info.nih.gov/ij/). For a randomly chosen subsample (P22R1_DT22; i.e. thermal selection population P22R1 reared at the developmental temperature of 22 °C) all wings from 30 females and 30 males were digitized and measured three times, which makes it possible to assess the measurement error caused by the imaging and digitizing (but not the mounting on slides) procedures (Palmer, 1994).

Larval competitive ability

First instar larvae from each thermal selection population were competed against larvae from the *ch-cu* marker strain, which is homozygous for the morphological recessive markers on the O chromosome *cherry eyes* (*ch*) and *curled wings* (*cu*). The competition assay was only carried out at the developmental temperature of 18 °C (the theoretical optimum for *D. subobscura*) because the

ch-cu strain has a long thermal selection regime at that temperature and its competitive ability at higher temperatures (22–23 °C) seems to be seriously hindered (M. Santos, unpublished observations). The competition vials were the same size as those used to measured egg to adult viability and development time under uncrowded larval conditions, and contained 4 mL of food. This amount of food is enough to keep humidity and hydration of the medium at 18 °C, and allows reducing the total amount of food available for the larvae. As in Partridge *et al.* (1994b) three larval densities were used: 33 wild plus 66 *ch-cu*, 66 wild plus 132 *ch-cu*, and 132 wild plus 264 *ch-cu*. Parenthetically, the larval density used to maintain all thermal populations is substantially lower: ~5 larvae/mL of food (Santos *et al.*, 2004). Eight replicate vials were set up for each thermal selection population and larval density, and the number of wild type and *ch-cu* adults emerging from each vial were recorded.

A useful measure of pre-adult competitive ability in each vial was obtained as:

$$v_i = \frac{a}{b+1} \times \frac{X_i}{Y_i},$$

where a and b are, respectively, the number of wild type and mutant flies raised, and X_i is the number of mutant first instar larvae added to the Y_i wild type larvae (v_i is an unbiased estimator of the viability of wild type larvae relative to *ch-cu*; see Santos *et al.*, 1992).

Statistical analyses

The unit of analysis here is the population, and the three replicated populations were treated as a random factor nested within thermal selection regime, which was a fixed effect in the linear models (see Sokal & Rohlf, 1995, p. 272).

Procrustes analysis

We used geometric morphometrics based on Procrustes methods (Bookstein, 1996; Dryden & Mardia, 1998; Zelditch *et al.*, 2004) to characterize wing size and shape variation in flies raised under uncrowded larval conditions. Procrustes superimposition extracts overall shape information (allometric and nonallometric components) from the original x and y coordinates after removing variation in scale, position and orientation. As measurements were taken for both wings, reflection was also eliminated by changing the sign of the x coordinate of every landmark for configurations from left wings (Klingenberg & McIntyre, 1998; Santos *et al.*, 2005b). All individuals of all configurations combined were superimposed simultaneously by a generalized least-squares Procrustes fit. The final iteration to minimize the sum of the squared distances between the landmarks of all wings in the sample was performed without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden & Mardia (1998; see also Rohlf, 1999). Scatterplots of superimposed Procrustes coordinates were visually inspected for gross outliers (e.g. mislabelling of landmarks) by using the 'plot' subroutine in MATLAB algebra program environment (V7.0.4, The MathWorks 2005a).

Wing shape variation

For each fly wing shape was estimated as the average Procrustes coordinates of left and right wings. In geometric morphometrics shape is defined as a character of the entire landmark configuration and, therefore, it is necessarily multivariate. With $p = 13$ landmarks, the dimensional space here is $2p–4 = 22$. This raises the problem that a multivariate analysis of variance (MANOVA) to the Procrustes coordinates as a simply extension to the ANOVA for (e.g.) centroid size could not test for thermal selection regimes as the appropriate error term is the variation among replicated populations and there are not enough degrees of freedom. To circumvent the problem we applied a two-way MANOVA with thermal selection and developmental temperature as fixed effects, and replicated populations nested in thermal selection as a random effect, to the retained 22 PC (principal components; Jolliffe, 1986) scores from the covariance matrix of the Procrustes coordinates but tested for shape variation among thermal selection populations by using a two-level nested Procrustes ANOVA. In this ANOVA the Procrustes sums of squares for each level were simply the sum of the sums of squares of separate two-level nested ANOVAS performed for each of the x and y coordinates of the aligned configurations (see Klingenberg & McIntyre, 1998). We then performed a randomization test to test the null hypothesis about main treatment effects in a randomized (i.e. random assignment) experiment (Edgington, 1995); namely, to test for among thermal regimes F-statistics random permutations among replicate and selection temperature were performed. Each test used 10 000 random permutations of the observations.

Developmental stability

Measurement error was assessed for the random subsample P22R1_DT22 that had been digitized three times through a conventional two-way mixed model ANOVA for centroid side, with sides as fixed and individuals as random factors (Leamy, 1984; Palmer & Strobeck, 1986), and the corresponding MANOVA extension for wing shape (Klingenberg *et al.*, 2002; Santos *et al.*, 2005b). For all other analyses we used the complete set of populations that had been digitized only once (the first measurement from subsample P22R1_DT22 was used in this case), which obviously means that the individual × side interaction and measurement error terms were grouped as a 'remainder'.

Computer software for statistical analysis

The computer programs used for statistical data analyses were MATLAB algebra program environment (V7.0.4,

The MathWorks 2005a) together with the collection of tools supplied by the STATISTICS TOOLBOX (V5.0.2, The MathWorks 2005b), and the statistical software packages STATISTICA V6 (2003) and SPSS V13 (2004). Some helpful functions in morphometrics from the MATLAB toolbox Res6 developed by R. E. Strauss (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm) were also used.

Results

Development times

A total of 9404 flies emerged from the 405 uncrowded experimental vials (mean ± SD: 23.22 ± 3.86). Development times from egg-lay to adult emergence for all thermal selection populations at each developmental temperature are plotted in Fig. 1. In each case the average time was calculated for each of the 15 replicated vials. The three-way ANOVA with thermal selection, developmental temperature and sex as fixed effects, and replicated populations nested in thermal selection as a random effect (Table 1), confirms that development times increased substantially with decreasing rearing temperature as expected. In addition, development times also varied significantly among thermal selection regimes but not between sexes, and Scheffé *post hoc* tests using the mean square from the replicated populations as the error term indicated that average development time for P13 populations (639.94 h) was significantly longer than those for P18 (610.26 h) and P22 (604.50 h), whose difference was, in turn, marginally nonsignificant ($P = 0.055$). From Fig. 1 it is also clear that P13 populations had longer development time at all developmental temperatures, whereas P18 and P22 populations took about the same time to develop at the highest developmental temperature and changed ranks at DT13 and DT18.

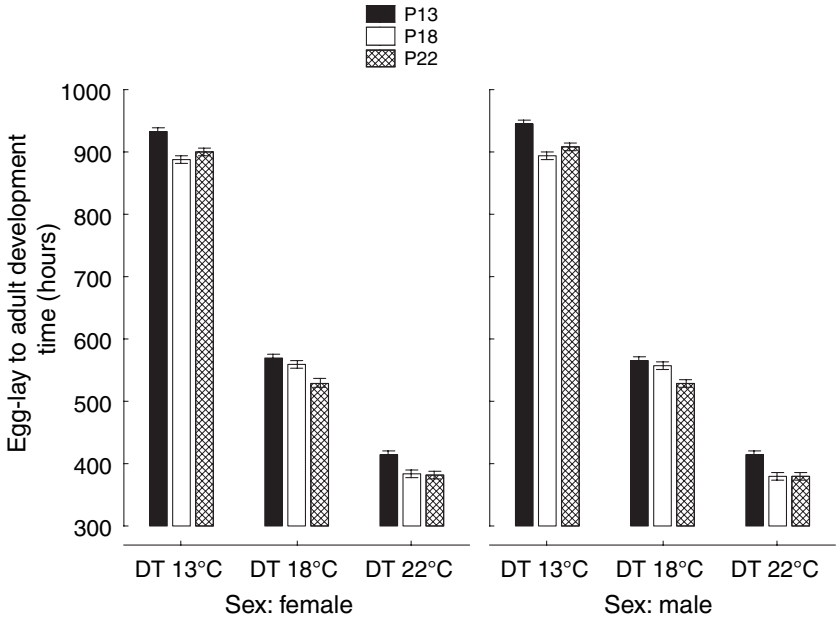

Fig. 1 Larval development time in uncrowded cultures: hours from egg-lay to adult emergence (means with 95% confidence intervals) for larvae from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled) reared at the three developmental temperatures (DT13, DT18 and DT22).

Source	d.f.	SS	MS	*F*	*P*
Thermal selection temperature	2	195250	97625	18.46	0.003
Populations within selection*	6	31726	5288	11.57	<0.001
Developmental temperature	2	38173271	19086636	41768.12	<0.001
Sex	1	451	451	0.99	0.321
Selection × developmental	4	51953	12988	28.42	<0.001
Selection × sex	2	256	128	0.28	0.756
Developmental × sex	2	5841	2920	6.39	0.002
Selection × developmental × sex	4	248	62	0.14	0.969
Error	786	359176	457		

*Error term for thermal selection temperature.

Table 1 Three-way cross-classification ANOVA on larval development time (in hours) from egg-lay to adult emergence (data plotted in Fig. 1).

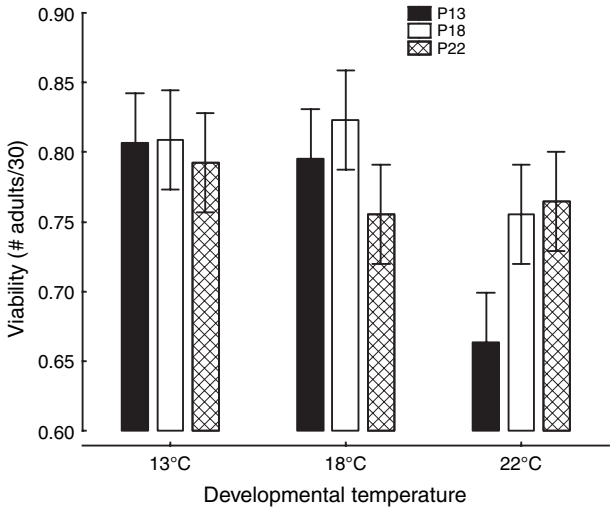

Fig. 2 Egg to adult viability in uncrowded cultures: viability (means with 95% confidence intervals) expressed as number of emerged adults/30 for each vial with an initial number of 30 eggs from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled) reared at the three developmental temperatures (DT13, DT18 and DT22).

After 81 generations of divergence, there was no evidence for cold- (P13) or warm-adapted (P22) populations to perform better at the developmental temperatures matching their past thermal selection regime. Thus, the average P13–P22 difference in development time was 35.04 h at DT13 and 32.97 h at DT22, and the between-population, between-developmental temperature contrast was not statistically significant ($F_{1,786} = 0.21$, $P = 0.647$). Inspection of the data also shows that females emerged slightly earlier than males at the lowest developmental temperature in all thermal selection regimes (11.51 h earlier for P13, 7.48 h for P18 and 8.26 h for P22), but sex differences vanished at DT18 and DT22.

Egg-to-adult viability under uncrowded larval conditions

Viability in each vial was recorded as the number of flies emerged divided by 30, which was the initial number of eggs. The 15 replicated vials were used to estimate the average viability for each thermal selection population, and results are plotted in Fig. 2. The two-way ANOVA with thermal selection and developmental temperature as fixed effects, and replicated populations nested in thermal selection as a random effect (Table 2), shows that average viabilities were significantly different among developmental temperatures, which was basically because of an overall reduction in viability of ~9% at the highest developmental temperature when compared with the average viabilities at DT13 and DT18. However, this reduction was clearly not uniform among thermal selection populations, which resulted in a highly significant selection × developmental temperature interaction effect. Scheffé *post hoc* tests indicated that viability of P13 populations significantly dropped (by ~21%) at DT22, while viabilities of P18 and P22 where about the same at all developmental temperatures.

Larval competitive ability

The total number of flies that emerged from the 216 vials was 14 292 (5659 wild type and 8633 *ch-cu*), which amounts to 28.64% (34.02% wild type and 25.95% *ch-cu*) of the initial number of first instar larvae. The replicated vials were used to estimate the average competitive ability of each thermal selection population (Fig. 3). The two-way ANOVA with thermal selection and larval crowding as fixed effects, and replicated populations nested in thermal selection regime as a random effect [using $\log_e(v_i)$ as the dependent variable], shows that competitive abilities were only significantly different among crowding conditions ($F_{2,201} = 24.91$, $P < 0.001$), with competitive ability dropping from low ($v_i = 1.57$) to medium ($v_i = 1.19$) or high ($v_i = 1.25$) larval crowding. However, no effect of selection temperature ($F_{2,6} = 0.51$, $P = 0.626$), or selection × crowding interaction ($F_{4,194} = 1.29$, $P = 0.276$), were detected.

Adult body size

For each fly wing size was estimated as the average centroid size of left and right wings, and the two females and two males sampled from each of the 15 replicated vials were used to estimate the average size for each thermal selection population (Fig. 4). The three-way ANOVA with thermal selection, developmental

Table 2 Two-way cross-classification ANOVA on egg to adult viability under uncrowded larval conditions (dependent variable was $\arcsin\sqrt{\frac{\text{Number of flies emerged}}{30}}$, where 30 is the initial number of eggs in the vial; data plotted in Fig. 2).

Source	d.f.	SS	MS	*F*	*P*
Thermal selection temperature	2	0.1988	0.0994	3.13	0.117
Populations within selection*	6	0.1903	0.0317	1.20	0.306
Developmental temperature	2	0.6304	0.3152	11.92	<0.001
Selection × developmental	4	0.3968	0.0992	3.75	0.005
Error	390	10.3125	0.0264		

*Error term for thermal selection temperature.

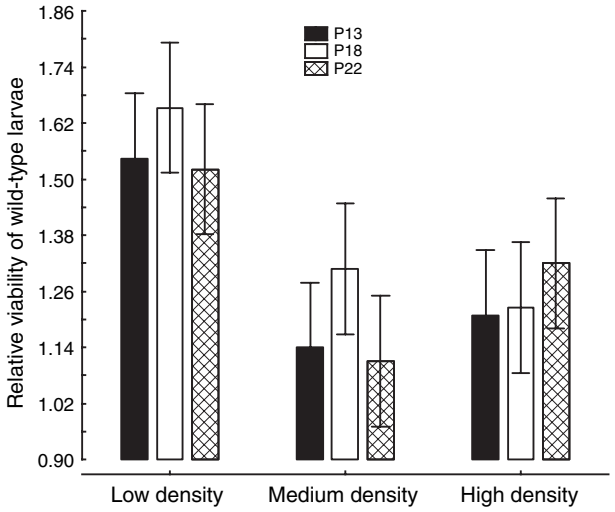

Fig. 3 Competitive survival ability at 18 °C: viability of wild type larvae relative to *ch-cu* tester stock (means with 95% confidence intervals) for larvae from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled) reared at three larval densities.

temperature and sex as fixed effects, and replicated populations nested in thermal selection as a random effect (Table 3), corroborates that wing size was strongly affected by developmental temperature as expected, with flies growing larger at lower rearing temperatures (a pattern known as the temperature-size rule; reviewed by Atkinson, 1994; Angilletta & Dunham, 2003). However, no statistically significant differences were detected among thermal selection regimes, indicating that cold-adapted flies were not bigger than their warm-adapted counterparts after 3.5 years of selection (81 generations of divergence). In addition, females were bigger than males as expected (10.22% bigger at DT13, 9.86% at DT18 and 10.82% at DT22) and the sex differences remained approximately constant at all rearing temperatures as no developmental temperature × sex interaction effect was detected. Finally, the statistically significant thermal selection × developmental temperature interaction effect simply reflects the different ranks of the thermal stocks at the three tested developmental temperatures: Scheffé *post hoc* tests did not detect any significant difference for wing size among the thermal selection regimes within developmental temperatures (results not shown). Therefore, the previously detected pattern after 2 years of thermal evolution, with smaller adults from P18 populations but no differences between cold- (P13) and warm-adapted (P22) stocks when flies were raised at 18 °C (Santos *et al.*, 2005a), seems to have vanished.

Wing shape

All effects from the MANOVAS on the Procrustes coordinates were highly significant, suggesting that overall wing shape varies significantly among thermal selection populations and developmental temperatures (Table 4). Scheffé *post hoc* tests revealed that wing shape was strongly affected by DT22 when compared with DT13 or DT18, which were only marginally significant for females ($P = 0.044$) but not for males ($P = 0.090$).

Principal component analyses were carried out for the group means of thermal populations and developmental temperatures, and the patterns of overall shape changes along PC1 (there are only two PCs for each main effect) are shown in Figs 5 and 6, respectively. There is a general narrowing (widening) of the wings as a consequence of

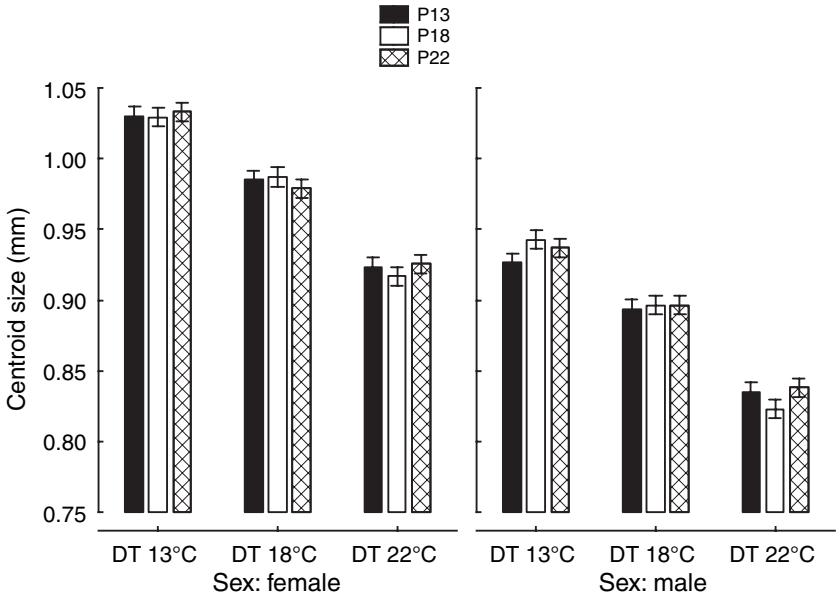

Fig. 4 Wing size in uncrowded cultures: centroid size was estimated from each fly in a normalized form (see Dryden & Mardia, 1998, p. 24) as the average of left and right wings, and the bars give the means (with 95% confidence intervals) of all flies sampled from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled) reared at the three developmental temperatures (DT13, DT18 and DT22).

Table 3 Three-way cross-classification ANOVA on centroid size (in $\log_e$ mm). (Data plotted in Fig. 4).

Source	d.f.	SS	MS	*F*	*P*
Thermal selection temperature	2	0.002917	0.001459	0.45	0.655
Populations within selection*	6	0.019264	0.003211	2.79	0.011
Developmental temperature	2	3.571782	1.785891	1551.99	<0.001
Sex	1	3.891460	3.891460	3381.78	<0.001
Selection × developmental	4	0.025505	0.006376	5.54	<0.001
Selection × sex	2	0.002577	0.001288	1.12	0.327
Developmental × sex	2	0.005212	0.002606	2.27	0.104
Selection × developmental × sex	4	0.009057	0.002264	1.97	0.097
Error	1596	1.836538	0.001151		

For each fly wing size was estimated as the average centroid size (in a normalized form; see Dryden & Mardia, 1998, p. 24) of left and right wings.
*Error term for thermal selection temperature.

Table 4 Two-way cross-classification MANOVAS for procrustes coordinates (22 PC scores) to test for thermal selection effects on overall wing shape variation and nonallometric shape variation.

	Females					Males				
Effect	Wilks' λ	*F*	d.f. 1	d.f. 2	*P*	Wilks' λ	*F*	d.f. 1	d.f. 2	*P*
Overall shape variation										
Thermal selection temperature	–	3.07	44	132	0.0086	–	4.68	44	132	0.0002
Populations within selection†	0.61254	3.00	132	4509	*	0.65695	2.56	132	4509	*
Developmental temperature	0.15312	54.73	44	1548	*	0.15134	55.25	44	1548	*
Selection × developmental	0.68472	3.50	88	3063	*	0.67481	3.64	88	3063	*
Nonallometric shape variation										
Thermal selection temperature	–	2.94	44	132	0.0091	–	3.74	44	132	0.0014
Populations within selection†	0.62164	2.91	132	4509	*	0.64380	2.69	132	4509	*
Developmental temperature	0.54207	12.60	44	1548	*	0.55264	12.14	44	1548	*
Selection × developmental	0.69069	3.41	88	3063	*	0.68707	3.47	88	3063	*

The nonallometric shape variation was tested from the residuals obtained from a multivariate regression of the procrustes coordinates on centroid size (in $\log_e$ mm). Thermal selection effects were tested by using a two-level nested procrustes ANOVA (see text for details).
†Error term for thermal selection temperature.
*$P < 0.001$

thermal selection and developmental temperature (recall that the direction of PCs is arbitrary and all the movements can be simultaneously reversed by 180°). Thus, the wing-aspect ratio estimated as $(\text{wing length})^2/\text{wing area}$ (see Azevedo *et al.*, 1998) significantly increased in P22 populations when compared with P13 and P18 (Fig. 7), which clearly suggest that the highest thermal environment selected for longer wings relative to wing width. On the other hand, the effect of rearing temperature on wing-aspect ratio was just the opposite to that of thermal selection; namely, wings were shorter relative to wing width at the highest developmental temperature DT22. Also prominent are the synchronized displacements of the cross-veins (anterior cross-vein is defined by landmarks 11 and 12; posterior cross-vein by landmarks 7 and 13) along their longitudinal veins according to developmental temperature (Fig. 6).

As could be expected from the strong effect of developmental temperature on wing size variation (Fig. 4 and Table 3), a multivariate regression of Procrustes coordinates on centroid size showed that most of the total shape variation in the complete data set was due to the allometric component. However, the MANOVAS using the residuals obtained from a multivariate regression of the Procrustes coordinates on centroid size (i.e. the shape variation independent of size) also detected a highly significant variation for all levels in the analysis (Table 4).

Developmental stability

Measurement error

Table 5 shows the conventional two-way mixed model ANOVA for centroid size and the MANOVA extension for wing shape carried out for the 30 females and 30 males digitized three times. The individual × side interaction, standing for FA, was highly significant, suggesting that measurement error does not seem to be a major concern for the subsequent analyses of FA in the thermal selection populations raised at the three developmental

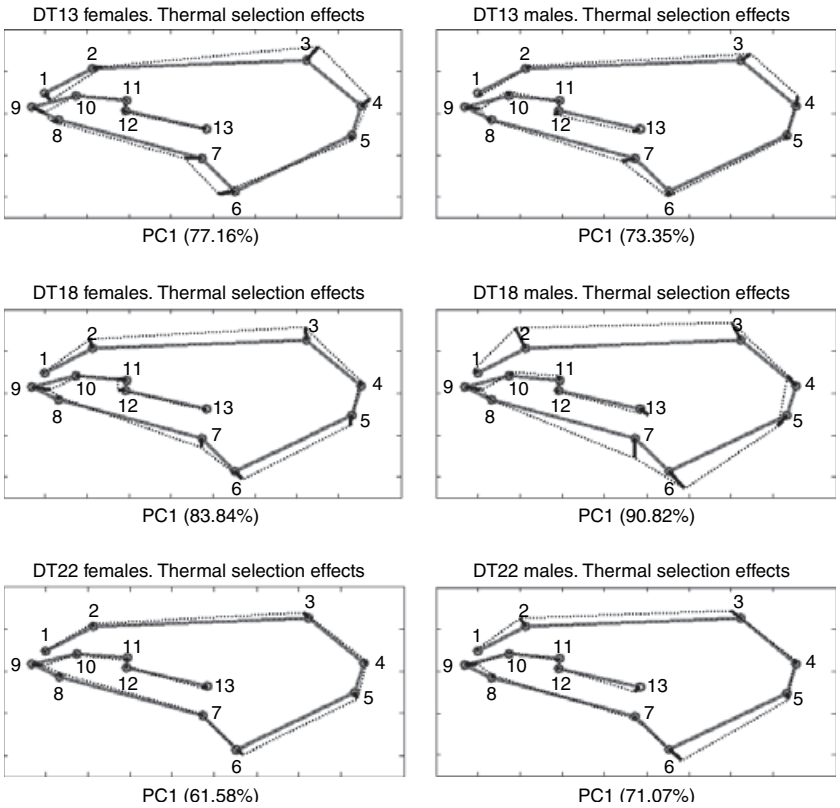

Fig. 5 Plots of landmark displacements from the consensus configuration (thick grey line: the average shape of the entire sample) attributable to the thermal selection regime. The dotted lines plot the PC1 for females and males at the three developmental temperatures (DT13, DT18 and DT22). landmark positions are indicated by numbers.

temperatures. Thus, the mean squares of the interaction term for centroid size exceeded the corresponding mean squares for the measurement error term by ~12 times. Similarly, the trace of the interaction covariance matrices of the Procrustes coordinates exceeded that for the corresponding measurement error covariance matrices by ~11 times. (Recall that the traces of the corresponding sum of squares and cross products matrices in the MANOVA extension for wing shape are just the sums of squares in the Procrustes ANOVA as implemented by Klingenberg & McIntyre, 1998).

Centroid size

Main effects ANOVAs with individuals and sides as main effects (wings were only digitized once and, hence, measurement error cannot be analysed separately) and centroid size (in $\log_e$ mm) as the dependent variable, were performed for all thermal selection populations (females and males separately; replicates pooled) raised at the various developmental temperatures (results not shown). In all cases but three (females from P22 raised at DT22, and males from P22 raised at DT18 and DT22) sides' effects were highly significant, with left wings bigger than the right ones in agreement with previous findings in *D. subobscura* (Fernández Iriarte *et al.*, 2003; Santos *et al.*, 2005b). A close inspection of the mean squares of the 'remainder' (FA plus measurement error) source of variation in the main effects ANOVAs revealed a clear pattern: wing size asymmetry (FA) was generally lower when flies were raised at DT18, the theoretical optimum for *D. subobscura* (Fig. 8).

A three-way ANOVA using the absolute value of FA scores (|left–right|) for centroid size (centroid size in $\log_e$ mm to remove linear size dependence for FA; e.g. Palmer & Strobeck, 2003) as the dependent variable, with thermal selection, developmental temperature and sex as fixed effects, and replicated populations nested in thermal selection as a random effect, only detected statistically significant differences for developmental temperature ($F_{2,1596} = 14.33$, $P < 0.001$), sex ($F_{1,1596} = 28.96$, $P < 0.001$), and their interaction ($F_{2,1596} = 4.67$, $P = 0.009$). In other words, there was no indication that centroid size FA has evolved as a result of thermal selection. The between-population, between-developmental temperature contrast suggests that cold- (P13) or warm-adapted (P22) populations performed approximately alike at the developmental temperature matching their past history of thermal selection when judge against the opposed extreme developmental temperature.

Wing shape

Main effects MANOVAs to the retained 22 PC scores of the Procrustes coordinates carried out for all thermal selection populations (females and males separately; replicates

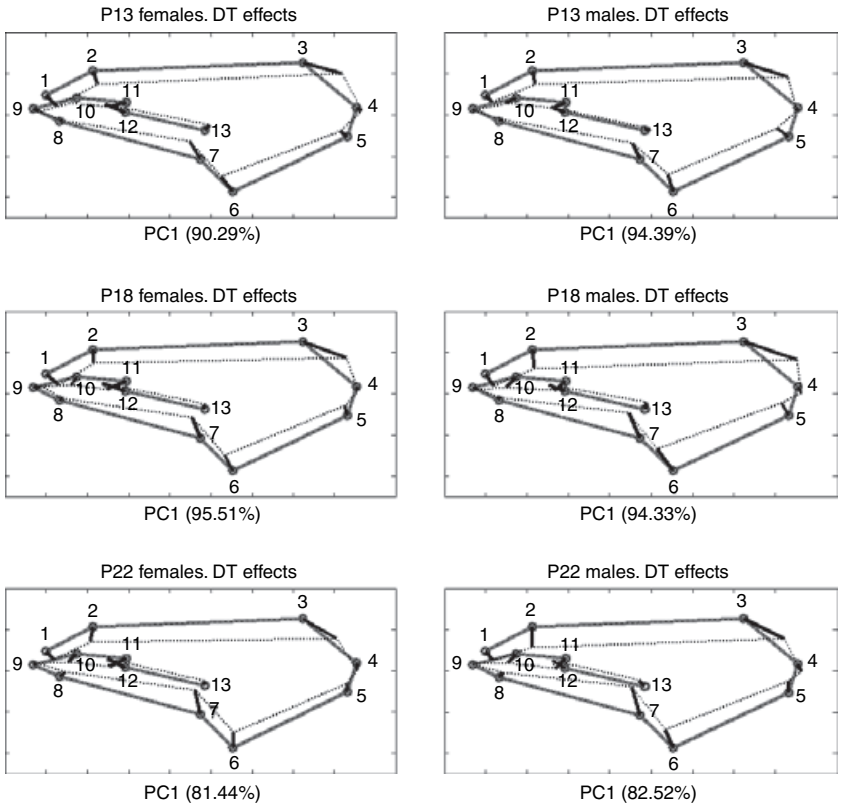

Fig. 6 Plots of landmark displacements from the consensus configuration (thick grey line: the average shape of the entire sample) attributable to developmental temperature (DT effects: DT13, DT18 and DT22). The dotted lines plot the PC1 for females and males from the three thermal selection populations (P13, P18 and P22). landmark positions are indicated by numbers.

pooled) raised at the various developmental temperatures revealed a highly significant sides' effect in all cases (results not shown). This uncovering of directional asymmetry for wing shape is a quite general finding when quantitative analyses of form are carried out using the powerful methods of geometric morphometrics to reveal even small morphological variation that otherwise would remain hidden with less effective techniques (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 1998; Santos *et al.*, 2005b).

To test for differences in wing shape FA between thermal selection regimes and developmental temperatures we took advantage of the following: (i) in dealing with random perturbations in development only the magnitude of those perturbations is generally of interest; and (ii) the sum of squares for the individuals × sides interaction in the Procrustes ANOVA as implemented by Klingenberg & McIntyre (1998) is straightforwardly related to the sum of Var(left–right) (index FA4 in Palmer, 1994) for each x and y coordinates of the corresponding aligned configurations divided by the shape dimension. Therefore, for each sex we estimated a 'FA4' index for wing shape in each replicated population from the 'remainder' (FA plus measurement error) source of variation in main effects Procrustes ANOVAS, with individuals and sides as main effects. These values were then used as the dependent variable in a three-way ANOVA with thermal selection, developmental temperature, and sex as fixed effects. Statistically significant differences were only detected for thermal selection regimes ($F_{2,36} = 12.18$, $P < 0.001$) and developmental temperatures ($F_{2,36} = 32.58$, $P < 0.001$) (Fig. 9). Scheffé *post hoc* tests indicated that wing shape asymmetry of P22 populations (2.267×10^{-5}) was significantly bigger than that for P13 (1.658×10^{-5}) and P18 (1.861×10^{-5}) populations, which did not differ between them.

To avoid potential problems with the underlying assumptions of the parametric ANOVA, a two-way randomization ANOVA exact test was also performed for each sex using the STATXACT (V.6, 2003) statistical package. These tests confirmed the previously detected differences for thermal selection regimes (females $P = 0.0153$; males $P = 0.005$) and developmental temperatures (females and males $P < 0.001$). Conversely to what had been found for centroid size, wing shape asymmetry increased with increasing developmental temperature and adaptation to a high temperature regime (Fig. 9).

Discussion

For pre-adult life history traits, the clearest patterns of evolutionary response to thermal selection *per se* exhibited by *D. subobscura* were the relatively low viability at the developmental temperature of 22 °C and the longer

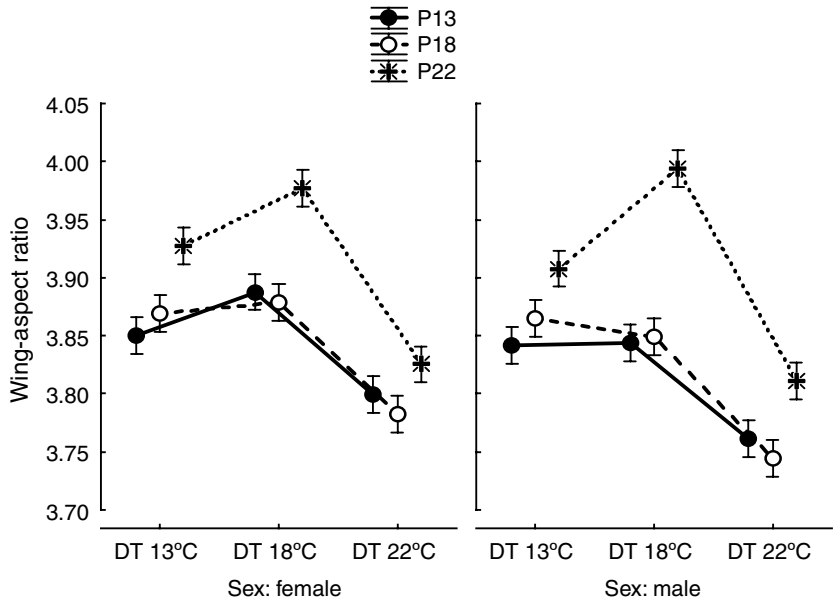

Fig. 7 Wing-aspect ratio estimated as (wing length)^2/wing area in flies emerged from the uncrowded cultures. Wing length was calculated as the combined lengths of the basal (i.e. the Euclidean distance between landmarks 9 and 13) and distal (Euclidean distance between landmarks 13 and 5) segments of longitudinal vein IV as it is customary in *D. subobscura* (e.g. Pegueroles *et al.*, 1995; Huey *et al.*, 2000; Gilchrist *et al.*, 2001), and wing area as the area of the polygon defined by the outer landmarks 2, 3, 4, 5, 6 and 9 by using the 'polyarea' subroutine in MATLAB algebra program environment (v7.0.4, the Mathworks, 2005a). (See Santos *et al.*, 2005b, http://www.biomedcentral.com/1471-2148/5/7, for an open access figure showing the landmarks used in this work.) The figure plots the means (with 95% confidence intervals) of all flies sampled from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled) reared at the three developmental temperatures (DT13, DT18 and DT22).

Table 5 Two-way ANOVAS [centroid size; MS values in $(\log_e \text{mm})^2 \times 10^4$] and MANOVAS (shape; 22 PC scores were retained) of *Drosophila subobscura* wings to assess measurement error from 30 females and 30 males whose two wings were digitized three times.

	Females			Males		
	d.f.	MS	*F*	d.f.	MS	*F*
Centroid size						
Individual	29	38.90	27.22**	29	23.96	9.43**
Side	1	2.98	$2.09^{\text{n.s.}}$	1	6.91	$2.72^{\text{n.s.}}$
Individual × side†	29	1.43	11.41**	29	2.54	13.05**
Measurement	120	0.13		120	0.19	
	d.f. 1	d.f. 2	Wilks' λ	d.f. 1	d.f. 2	Wilks' λ
Wing shape						
Individual	638	243	2.24×10^{-17}**	638	243	7.53×10^{-18} **
Side	22	8	$0.153^{\text{n.s.}}$	22	8	0.046*
Individual × side	638	1842	8.39×10^{-15} **	638	1842	2.03×0^{-13} **

† Error term for individual and side.
$^{\text{n.s.}}P > 0.05$; *$P < 0.01$; **$P < 0.001$

development time at all developmental temperatures for the cold-adapted (P13) populations. The results also suggest that warm-adapted (P22) populations may have evolved faster development. Viability was the only trait that showed some evidence of beneficial adaptation in that for each thermal selection line it tended to be highest at the developmental temperature at which the stock was selected. For wing traits the results confirm and extend the previous observations after one and 2 years of thermal evolution (Santos *et al.*, 2004, 2005a) in that no divergence for wing size was detected following adaptation to temperature extremes (13 and 22 °C), but wing shape changes were clear as a result of both thermal adaptation and development at different temperatures. In

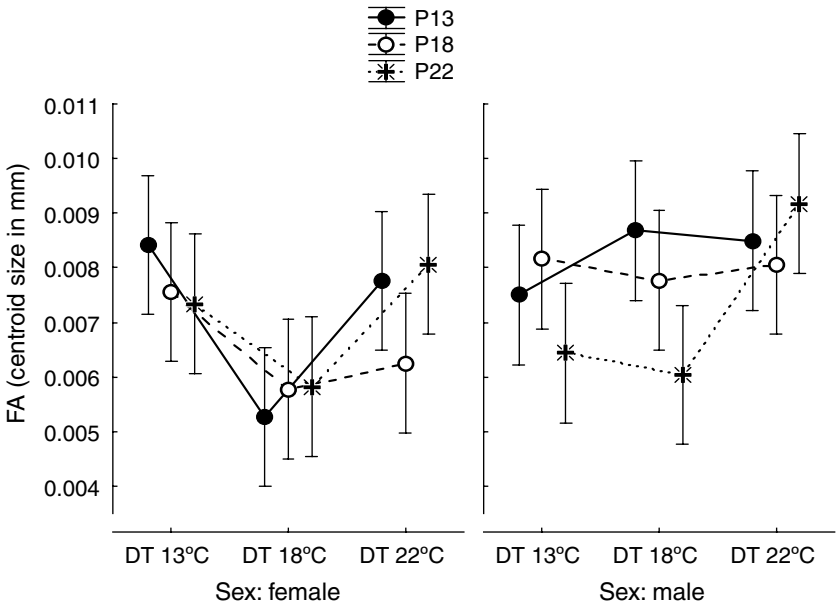

Fig. 8 Unsigned asymmetry (FA plotted as the absolute difference of left–right wing centroid size in millimetre; means with 95% confidence intervals) from all thermal selection populations (P13, P18 and P22; the three replicated populations within each thermal selection regime were pooled in the analyses) reared at the three developmental temperatures (DT13, DT18 and DT22).

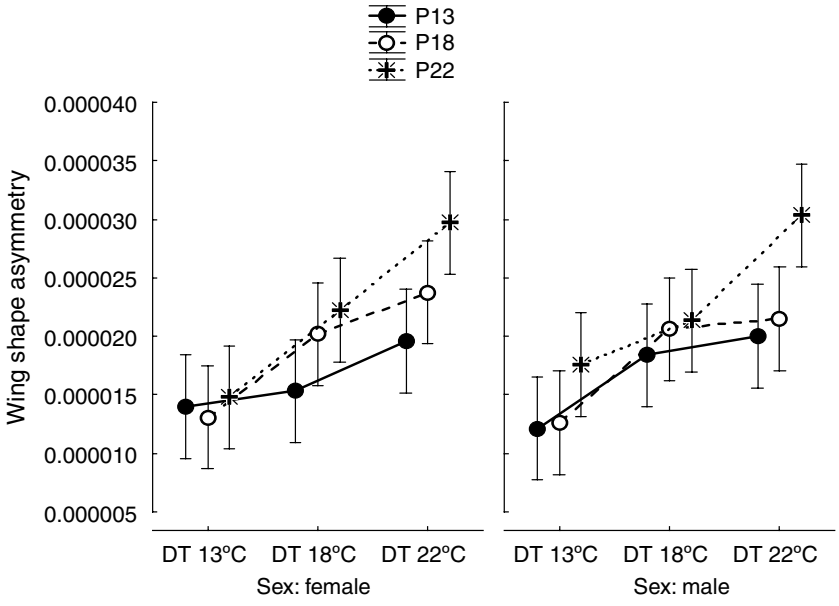

Fig. 9 Wing shape asymmetry (means with 95% confidence intervals) as a function of developmental temperature: a 'FA4' index for each replicated population was estimated as the mean square of the 'remainder' source of variation in main effects Procrustes ANOVAS (see text for details).

addition, changes in asymmetry of wing size and wing shape are observed according to temperature of development, and there was some indication that wing shape asymmetry has evolutionarily increased in warm-adapted (P22) populations. This clearly suggests that there is additive genetic variation for wing shape FA in *D. subobscura*.

Our results after 3.5 years of thermal evolution are in sharp contrast to those previously obtained with *D. melanogaster*, where cold-adapted (16.5 °C) populations evolved larger body size and grew faster partially as a result of greater efficiency of food use than their warm-adapted (25 °C) counterparts (Partridge *et al.*, 1994b; James & Partridge, 1995; Neat *et al.*, 1995), thus violating the somewhat standard within-population association between large adult body size and slow larval development (e.g. Santos *et al.*, 1994; Betrán *et al.*, 1998). Contrary to the widely held view that a faster development increases fitness in *Drosophila*, recent theoretical and laboratory studies suggest that fast growth carries a fitness cost (see e.g. Blanckenhorn, 2000). Some evidence from *D. melanogaster* indicates that adaptation to larval crowding increases larval feeding-rate with an associated reduction of growth efficiency (i.e. conversion of food into adult body mass;

Joshi & Mueller, 1996; Santos *et al.*, 1997), and that a fast growth may decrease adult longevity in populations reared under controlled larval densities (Chippindale *et al.*, 1994; also see Zwaan *et al.*, 1995). In this vein the lower larval survival of *D. melanogaster* cold-adapted populations when tested at 25 °C was tentatively attributed to a cost of fast growth (Partridge *et al.*, 1994b). Similarly, their relative survival also declined as larval rearing density increased, suggesting a trade-off between efficiency at converting food into biomass and larval competitive ability. However, this finding could be partially explained as due to the past history of adaptation to relatively low levels of larval crowding in their cold-adapted populations as *D. melanogaster* stabilize at lower densities when maintained at lower temperatures. Thus, some experimental evidence indicates that populations adapted to larval crowding show higher pre-adult survivorship when assayed at high densities (Mueller, 1988), but their growth rate relative to control populations interacts with crowding conditions with faster development at high and no differences at moderate densities (Santos *et al.*, 1997; Borash & Ho, 2001).

Certainly the costs and benefits of fast growth and larger body size may be context dependent, including the past evolutionary history of individuals. Interestingly, lines derived from the 25 °C thermal selection lines (the optimum temperature for *D. melanogaster*) and subsequently reared at 29 °C for 4 years showed no divergence in development time from the 25 °C lines, and the reason for this lack of change was not clear (James & Partridge, 1995). The outcome of laboratory thermal adaptation on body size and development time in *D. melanogaster* was also investigated by Bochdanovits & de Jong (2003). They performed a factorial design by using a combination of low (17.5 °C) and high (27.5 °C) thermal selection regimes and two different levels of food (poor and standard), and assayed the populations after 10 generations at both experimental temperatures under standard food conditions. A highly significant interaction between thermal and food level adaptation was detected for body mass, with no statistically significant differences for main effects when flies were tested at the experimental temperature of 17.5 °C but larger males emerged from cold-adapted populations when flies were assayed at 27.5 °C. On the other hand, thermal evolution did not bring about a difference in development time. They concluded that resource allocation at low food levels trades-off with thermal adaptation: a cold environment leads to increasing adult size whereas a warm environment leads to increasing larval survival. Our present thermal selection experiments were performed at standard food levels (see Santos *et al.*, 2004) and have also detected decreased larval survival in cold-adapted populations when tested at 22 °C (Fig. 2) associated with their longer development time (Fig. 1), which cannot obviously be attributed to a trade-off between body size and survival as no divergence in wing size as a result of thermal adaptation was observed (Fig. 4). The lack of divergence for larval competitive ability (Fig. 3) confirms that thermal selection did not bring about marked differences in the way resources were obtained and utilized. The cold-adapted *D. subobscura* stocks seem to have achieved the 'target' size by growing more slowly, and this could have led to just the opposite cost for survival at high temperatures than the one observed by Partridge *et al.* (1994b) in their thermal lines.

Taken together, laboratory studies on thermal adaptation fit into an interesting but somewhat foggy picture of how resource levels interact with pre-adult life history traits and adult body size. Food quality was not experimentally manipulated in Partridge *et al.* (1994a, b); however, covariation between the thermal selection regime and crowding conditions is likely to have brought about a combination of poor food quality, high levels of harmful waste products and juvenile mortality at high temperature, and 'good enough' food quality with low levels of juvenile mortality at low temperature. The higher juvenile mortality in their warm-adapted populations should favour a reduction in age a maturation which, in turn, would produce a correlated decrease in body size (e.g. Roff, 1981).

Gilchrist & Huey (2004) have recently used *D. subobscura* to test the idea that size itself is the target of selection by looking at the relationship between wing area and thorax length along two latitudinal genetic clines in body size; one from the original Palearctic region and the other from South American colonizers. Flight performance is influenced by size and the adaptive expectation is a higher wing:thorax size ratio at low temperatures (also a higher wing-aspect ratio; see Azevedo *et al.*, 1998). They found a consistent adaptive plastic response (i.e. larger wings at lower developmental temperatures), but mixed results according to the source of the populations; namely, wing: thorax size ratio increased with increasing latitude in Europe (in harmony with the adaptive hypothesis of evolutionary change) but decreased with increasing latitude in South American populations (consistent with a simple dimensional expectation). Our results from the thermal selection stocks founded with flies coming from the epicentre of the original New World invasion (Puerto Montt, Chile) are generally consistent with the findings of Gilchrist & Huey (2004) for the South American flies. Thus, we have detected a plastic response with a lower wing-aspect ratio at the highest developmental temperature, but warm-adapted populations evolved a higher wing-aspect ratio in contrast to the adaptive expectation (Fig. 7). Together with the lack of genetic differentiation for centroid size in the thermal stocks, the general conclusion is that wing size itself is not the target of thermal selection. A caveat: our controlled experimental conditions did not obviously mirror adult performance under natural conditions and might have been unsuccessful at identifying the real impact of temperature on wing size.

The wing shape asymmetry data presented here clearly suggest that this trait can evolve as a response to the thermal environment. Previous analyses using isochromosomal lines of *D. subobscura* did not detect any additive genetic variation of wing shape FA (Santos *et al.*, 2005b), which is not surprising in view of the very low values for the heritability of FA observed in most studies (Santos, 2002; Fuller & Houle, 2003). As discussed by Leamy & Klingenberg (2005) it is quite likely that no genes govern FA *per se* but FA levels are probably character-specific and influenced by dominance or epistatic interactions. According to these authors the consistently low levels of FA heritability suggest that any additive genetic variation generated by epistasis is continually eroded under normal circumstances; however, adaptation to rapid changes caused by various stresses may generate the conversion of epistasis into additive genetic variation in FA. We add the caveat that a low heritability of FA is compatible with a large enough evolvability of developmental stability to fuel an evolutionary response (see Pélabon *et al.*, 2004). The evolutionary increase of wing shape FA in warm-adapted (P22) populations is evidence of a strong effect of directional selection on developmental stability, likely as a result of thermal stress. This increase in FA was not accompanied by a simultaneous increase in wing size (Fig. 4), or in phenotypic variation for centroid size or overall wing shape (results not shown). We could, therefore, speculate that thermal effects on growth rate (warm-adapted populations seem to have evolved faster development) have resulted in a cost to the degree of developmental precision (see Calow, 1982), which obviously does not imply that wing shape FA *per se* has anything to do with fitness differences. Assuming that FA is unlinked to fitness, the standing higher FA level in P22 populations is compatible with a transient increase of FA during rapid adaptation to a warm environment.

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