

INTEGR. COMP. BIOL., 44:461–470 (2004)

Plastic and Genetic Variation in Wing Loading as a Function of Temperature Within and Among Parallel Clines in *Drosophila subobscura*¹GEORGE W. GILCHRIST^{*2} AND RAYMOND B. HUEY[†]^{*}Department of Biology, Box 8795, College of William & Mary, Williamsburg, Virginia 23187-8795[†]Department of Biology, Box 351800, University of Washington, Seattle, Washington 98195-1800

SYNOPSIS. *Drosophila subobscura* is a European (EU) species that was introduced into South America (SA) approximately 25 years ago. Previous studies have found rapid clinal evolution in wing size and in chromosome inversion frequency in the SA colonists, and these clines parallel those found among the ancestral EU populations. Here we examine thermoplastic changes in wing length in flies reared at 15, 20, and 25°C from 10 populations on each continent. Wings are plastically largest in flies reared at 15°C (the coldest temperature) and genetically largest from populations that experience cooler temperatures on both continents. We hypothesize that flies living in cold temperatures benefit from reduced wing loading: ectotherms with cold muscles generate less power per wing beat, and hence larger wings and/or a smaller mass would facilitate flight. We develop a simple null model, based on isometric growth, to test our hypothesis. We find that both EU and SA flies exhibit adaptive plasticity in wing loading: flies reared at 15°C generally have lower wing loadings than do flies reared at 20°C or 25°C. Clinal patterns, however, are strikingly different. The ancestral EU populations show adaptive clinal variation at rearing a temperature of 15°C: flies from cool climates have lower wing loadings. In the colonizing populations from SA, however, we cannot reject the null model: wing loading increases with decreasing clinal temperatures. Our data suggest that selective factors other than flight have favored the rapid evolution of large overall size at low environmental temperatures. However, selection for increased flight ability in such environments may secondarily favor reduced body mass.

INTRODUCTION

Most ectotherms show thermally mediated plasticity in body size: animals that develop at a lower temperature generally reach maturity at a larger size (Atkinson, 1994). Likewise, many studies of *Drosophila* show clinal (genetic) variation in wing and thorax size, with larger flies generally found in cooler habitats (Stalker and Carson, 1947; Sokoloff, 1965; David and Bocquet, 1975; Coyne and Beecham, 1987; Capy *et al.*, 1993; Imasheva *et al.*, 1994; James *et al.*, 1995; Pegueroles *et al.*, 1995; van't Land *et al.*, 1995). Repeated patterns of clinal variation in body and wing size of *Drosophila* and other insect species provides a remarkable testament to the power of natural selection (Endler, 1977), although exactly what is being selected remains elusive (Partridge and French, 1996; Bochdanovits and de Jong, 2003; Santos *et al.*, 2004)!

Two classes of adaptive explanations and one class of nonadaptive hypotheses for thermal influences on body size have been proposed. The first adaptive class suggests that size itself is under selection at high and/or low temperatures (Partridge and French, 1996). Flies that were artificially selected for large winged adults (while controlling for cell size changes) had higher fitness under cold conditions than flies selected for small wing size in both female (McCabe and Partridge, 1997) and male (Reeve *et al.*, 2000) *Drosophila melanogaster*. Relatively large females and males

show a prolonged survivorship and higher fecundity under cold conditions, although the precise mechanisms conferring higher fitness to large winged individuals in the cold were unknown. One possibility is large wing size increases flight ability at cool temperatures and this, in fact, is what is under selection. Mechanistically, this would require that wing size, but not body mass, would increase in the cold. This idea will be developed more fully below.

The second class of adaptive explanation argues that size changes with regards to temperature are a correlated effect of natural selection acting on the thermal sensitivity of life history characters. For example, if juvenile mortality in warm environments is high, then selection should favor a reduction in age at maturation, with a correlated reduction in body size (*e.g.*, Williams, 1966; Roff, 1981; Ludwig and Rowe, 1990). Variants of the correlated model include a possible overall advantage of short generation time when temperatures are high and resources are abundant, driven by “interest compounding” population growth (*e.g.*, Sibley and Atkinson, 1994). Nunney (1996) showed that selection for a shorter larval development time in *D. melanogaster* produced a 15% reduction in adult weight. Most of these models predict an overall reduction in body mass as a consequence of selection for reduced development time.

Finally, nonadaptive models, which suggest that changes in size with temperature arise as an incidental effect of growth and developmental constraints, have been used to predict both clinal and plastic increases in body size at lower temperatures (von Bertalanffy, 1960; van der Have and de Jong, 1996; Van Voorhies,

¹ From the Symposium *Evolution of Thermal Reaction Norms for Growth Rate and Body Size in Ectotherms* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 5–9 January 2004, at New Orleans, Louisiana.

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1996). These models predict an increase in overall body mass with declining temperature to some critical point at which mass will begin to decline with further decreases in temperature.

The relative size of flying animals has an important functional significance: the flight apparatus must be able to overcome the force of gravity and lift the mass of the body. The flapping motion of the wings greatly complicates the aerodynamics, compared to a fixed wing operating under steady state conditions, but some generalities can still be drawn. The amount of lift generated by a single wing stroke is positively related to the area, shape, and velocity of the wing (Dudley, 2000). Assuming a constant wing shape and oscillating stroke, the average lift is proportional to the square of wingbeat frequency (Ellington, 1984). Because the wingbeat frequency and power output of small ectotherms, like *Drosophila*, declines with decreasing temperature (Curtsinger and Laurie-Ahlberg, 1981; Unwin and Corbet, 1984; Stevenson and Josephson, 1990), the total lift generated by flies exposed to cold temperatures declines substantially.

One way to compensate for these effects of low temperature on wingbeat frequency would be to increase the relative size of the wings or, more specifically, to reduce the overall wing loading (Reed *et al.*, 1942; Stalker, 1980; Starmer and Wolf, 1989; David *et al.*, 1994; Azevedo *et al.*, 1998). Wing loading (ρ_w) is defined as the pressure exerted by the wings on the surrounding air: $\rho_w = \text{wet weight/wing area}$. All else being equal, either a plastic or genetic reduction of wing loading under cool conditions would facilitate flight performance. Indeed, Stalker (1980) found that *Drosophila melanogaster* that were capable of flight between 13 and 15°C had lower values for an index proportional to wing loading than did flies that were able to fly only at warmer temperatures (16 and 28°C).

On the other hand, simple dimensional analysis of the above equation yields the expectation that wing loading will increase with overall size (Dudley, 2000). For a characteristic linear dimension l , wing loading should increase with size: $\rho_w \propto l^3/l^2 = l$. Indeed, wing loading does increase with body mass in broad taxonomic comparisons of various species of moths (Casey and Joos, 1983) and drosophilids (Starmer and Wolf, 1989). Within species, however, size (l) generally increases either genetically or plastically with decreasing temperature; thus the null expectation is that wing loading also should increase in animals adapted to or grown in cold conditions. The contrast between this null model, predicting *greater wing loading in the cold versus the counter-gradient (adaptive) model* (Conover and Present, 1990) of *reduced wing loading in the cold* outlined above provides a well defined set of qualitative predictions for patterns of plastic and clinal variation (Fig. 1). Assume that wing size a is negatively related to developmental temperatures (T_{dev}), such that animals reared at high temperatures have smaller wings than those reared at low temperatures and that b) wing size is genetically and inversely related to clin-

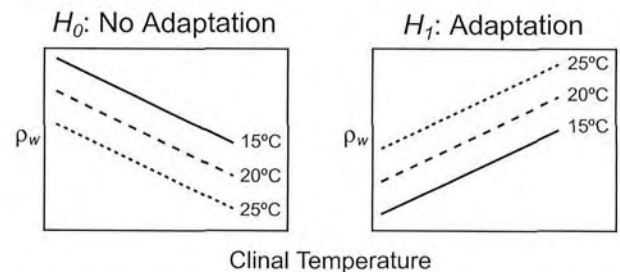


FIG. 1. Null and adaptive hypotheses for changes in wing loading (ρ_w) with temperature. T_{dev} is indicated by the temperatures on the right.

al temperature patterns (T_{cline}), such that animals adapted to warm conditions evolve smaller wings than those adapted to cool environments. Then we can propose the following hypotheses:

For plastic variation in response to T_{dev} :

H_0 : **Null hypothesis:** wing loading should scale negatively with rearing temperature (T_{dev}). Flies that develop in cooler conditions should have higher wing loadings than those that develop in warmer environments (compare the *positions* of the lines in Fig. 1, left).

H_1 : **Adaptive plasticity:** flies developing in cooler conditions should have lower wing loadings than those developing in warmer environments (*positions* of lines, Fig. 1, right).

For clinal (genetic) variation in response to T_{cline} :

H_0 : **Null hypothesis:** wing loading should scale negatively with T_{cline} ; large flies from cool habitats should have higher wing loadings than flies from warm habitats (compare the *slopes* of the lines in Fig. 1, left).

H_1 : **Adaptive clines:** wing loading should scale positively with T_{cline} . Large flies from cool climates should have lower wing loadings than those from warm habitats (*slopes* of the lines in Fig. 1, right).

In this paper, we examine both plastic and clinal changes in wing length and wing loading in native (European) and introduced (South American) populations of *Drosophila subobscura* along parallel latitudinal gradients that span ~1,500 km. Size clines are well documented in the ancestral European populations (Prevosti, 1955; Misra and Reeve, 1964). The introduction of *D. subobscura* to South and North America in the late 1970s (Brncic and Budnik, 1980; Beckenbach and Prevosti, 1986; Ayala *et al.*, 1989) set up a remarkable “natural experiment” in evolution. Collections about a decade after the colonization failed to detect a cline in wing size (Pegueroles *et al.*, 1995). However, collections two decades after the introduction show that both North (Huey *et al.*, 2000; Gilchrist *et al.*, 2001) and South American (Gilchrist *et al.*, 2004) populations had evolved size clines parallel to those in Europe. We survey the descendants of flies collected from 11 sites in EU and 10 sites in SA. Lab

populations were reared under uniform conditions for approximately 10 generations. Individuals from each population were then reared from egg to adulthood at 15, 20 or 25°C to assess plastic variation in body size. As rearing conditions within each T_{dev} are the same for all populations, we interpret variation among populations as reflecting clinal patterns of genetic variation. Ambient temperatures on both continents are inversely correlated with latitude (Table 1).

We tested our hypotheses with a series of linear models (outlined below). We find that both the ancestral European and the recently invading populations in South America exhibit adaptive plasticity: wings are larger for flies reared in the cold, even though wing loading is reduced. Thus, the new colonists share that ancestral pattern of plastic variation. Clinal patterns differ, however, between continents. European flies exhibit either a flat or a positive clinal pattern of wing loading with regard to temperature. This is consistent with the adaptive hypothesis for evolutionary change. However, we cannot reject the null clinal hypothesis for the South American flies: wing loading increases genetically with body size and with decreasing environmental temperature. Thus we see consistent patterns of thermoplastic variation in both the ancestral and derived populations, but only adaptive clinal change in the ancestors.

METHODS AND MATERIALS

The flies used in this study are descended from European collections made in April 1998 and South American collections made in November 1999. Collecting sites are listed in Table 1. At each site 20 to 25 gravid females were collected and maintained in the lab as isofemale lines for about five generations. Ten males and ten females were collected from each isofemale line and combined in a population cage maintained under continuous culture in a Percival incubator at 20°C on 14L:10D. Populations are maintained at approximately 1,000 adults on cornmeal/molasses/yeast medium.

Flies for these experiments were established from the lab populations in the summer of 2000 (Europe) and the summer of 2001 (SA). Eggs were collected from the cages on yeasted plates and transferred in groups of 50 to vials containing 10 ml of medium. 12 vials were collected for each population; four replicate vials were transferred to each developmental temperature (15, 20, or 25°C) and reared to adulthood. Because of the large number of measurements that had to be recorded on each individual, populations within each continent were staggered in randomly chosen groups of three at two- to three-week intervals.

Adults were held for about 24 hr after eclosion before being killed with an overdose of ether. They were immediately weighed and assigned a unique identification label before being placed individually into 0.5 ml microcentrifuge tubes and frozen at -4°C. Within a few days, the thorax was photographed in lateral view through an Olympus SZX12 dissecting micro-

TABLE 1. Source sites for flies used in this study and weather stations used for constructing the Clinal Temperature Index (T_{clim}).*

| Pop | Loc | Lat | Long | Alt | Country | Weather Station | Lat | Long | Alt | T_{clim} |
|----------------|-----|---------|---------|-----|-------------|------------------------|---------|---------|-----|------------|
| Aarhus | AA | 56°9' | 10°13'E | 0 | Denmark | Griben | 56°0' | 11°17'E | 4 | -2.728 |
| Leeds | LE | 53°48' | 1°34'E | 63 | UK | Bingley | 53°49' | 1°52'E | 262 | -3.489 |
| Leiden | LN | 52°9' | 4°30'E | -1 | Netherlands | Valkenburg | 52°11' | 4°25'E | 1 | -2.085 |
| Lille | LI | 50°38' | 3°4'E | 24 | France | Lille | 50°34' | 3°6'E | 47 | -1.898 |
| Gif-sur-Yvette | GF | 48°44' | 2°8'E | 127 | France | Grouin De Cancale | 48°43' | 1°51'W | 40 | -0.472 |
| Dijon | DI | 47°21' | 5°1'E | 235 | France | Dijon | 47°16' | 5°5'E | 222 | -1.773 |
| Lyon | LY | 45°31' | 4°50'E | 260 | France | Lyon/Satolas | 45°44' | 5°5'E | 248 | -1.038 |
| Montpellier | MO | 43°38' | 3°53'E | 18 | France | Montpellier | 43°35' | 3°58'E | 5 | 1.568 |
| Valencia | VA | 39°26' | 0°22'W | 7 | Spain | Valencia | 39°29' | 0°23'W | 13 | 4.482 |
| Malaga | MA | 36°45' | 4°25'W | 0 | Spain | Malaga/El Rompedizo | 36°40' | 4°29'W | 8 | 4.629 |
| Coyhaique | CQ | -45°35' | 72°4'W | 302 | Chile | Coyhaique | -45°35' | 72°7'W | 310 | -2.509 |
| Castro | CA | -42°30' | 73°46'W | 0 | Chile | Ancud | -41°54' | 73°48'W | 11 | -1.112 |
| Puerto Montt | PM | -41°28' | 72°56'W | 0 | Chile | Puerto Montt/El Tepual | -41°26' | 73°5'W | 85 | -1.278 |
| Valdivia | VD | -39°46' | 73°14'W | 4 | Chile | Valdivia | -39°39' | 73°5'W | 53 | -0.606 |
| Laja | LJ | -37°10' | 72°42'W | 49 | Chile | Concepcion | -36°46' | 73°3'W | 12 | 0.510 |
| Chillan | CH | -36°37' | 72°7'W | 129 | Chile | Chillan/O'Higgins | -36°35' | 72°2'W | 148 | 1.336 |
| Curico | CU | -34°55' | 71°14'W | 214 | Chile | Curico | -34°58' | 71°13'W | 225 | 1.055 |
| Santiago | SG | -33°30' | 70°40'W | 521 | Chile | Santiago/Pedahuel | -33°23' | 70°53'W | 480 | 1.362 |
| Illapel | IP | -32°0' | 71°10'W | 388 | Chile | Quintero | -32°47' | 71°31'W | 3 | 1.315 |
| LaSerena | LS | -29°55' | 71°15'W | 28 | Chile | Forada | -29°55' | 71°14'W | 137 | 2.026 |

* Loc = locality index, Lat = latitude, Long = longitude, Alt = altitude. More details are available in Gilchrist et al. (2004).

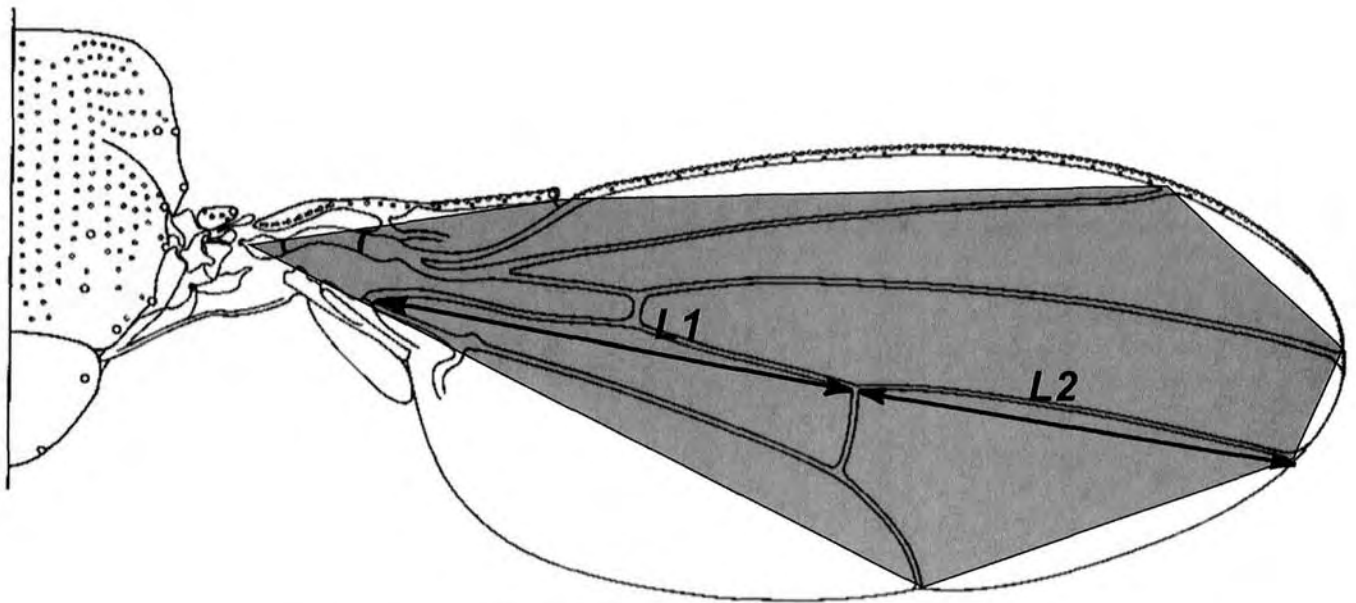


FIG. 2. Wing area index (shaded grey) and wing length (the sum of $L1$ and $L2$).

scope with a Fujix HC-300Z digital camera. Both wings were removed at their insertion point on the thorax and mounted on a microscope slide. The left and right wings were digitally photographed individually. Thorax length (from the anterior edge of the pronotum to the posterior tip of the scutellum) was digitally recorded (SigmaScan, SPSS, Inc. Chicago). Several landmarks on each wing (Fig. 2) were also digitized, and the area of their convex hull was computed as an index of wing area. Wing length was computed as the summed distances $L1$ and $L2$, which are the proximal and distal segments of the IVth wing vein (Fig. 2).

Statistical analysis

For each population and sex, we analyze the mean of wing length, wing loading, wing area, and wet mass. Clinal patterns were determined by linear regression of the trait means on clinal temperature (T_{cline}). To analyze both plastic and clinal patterns of wing length or wing loading, we used a general linear model with developmental temperature (T_{dev}) as an ordered factor, clinal temperature (T_{cline}) as a continuous variable, and Continent and Sex as fixed factors. The general model is $Trait \sim Continent + Sex + T_{dev} + T_{cline} + interactions + error$. Initially, all interactions were computed to test for homogeneity of linear effects. All non-significant interaction terms were removed for the final models. The clinal temperature index (T_{cline} , the first principal component of mean seasonal minimum and maximum daily temperatures) was created from published weather data for each site and shows a strong negative correlation with latitude on both continents (Table 1; for more information, see Gilchrist *et al.*, 2004). Specific criteria for rejecting the various null models are detailed in the Results.

RESULTS

Does wing size increase in the cold?

Previous studies suggest that we should observe plastic increases in wing length with decreasing development temperature: this would be apparent if we see a significant negative linear orthogonal polynomial contrast for T_{dev} (Huey *et al.*, 1999). Heterogeneity among the sexes or the continents would be indicated by a significant interaction between T_{dev} and Sex or Continent. Inspecting the relative positions of the regression lines in Figure 3, we note that wing size decreases with increasing T_{dev} for both females and males in both ancestral Europe and recently invaded South America (linear coefficient: -0.1063 , $F_{[1, 120]} = 2382$, $P < 0.0001$). We found no significant interaction terms (all $P > 0.10$). Furthermore, overall wing length between Europe and SA was not significantly different ($F_{[1, 120]} = 2.24$, $P > 0.1$).

Clinal (genetic) patterns in size would be indicated by a significant negative slope of wing length on T_{cline} , such that flies from cool temperature sites would be significantly larger than those from warm temperature sites. The regressions for each combination of Continent, Sex, and T_{dev} are shown in Figure 3, and the statistics tabulated in Table 2. At the near-optimal temperature of 20°C (Maynard-Smith, 1957; Maynard-Smith, 1958), both sexes on both continents show significant clinal decreases in wing length with temperature. Interestingly, the clinal pattern is somewhat reduced in the colonizing South American flies that were reared at either 15 or 25°C (slopes not significantly different from zero), whereas the ancestral European populations show parallel clines at all rearing temperatures (Fig. 3, Table 2).

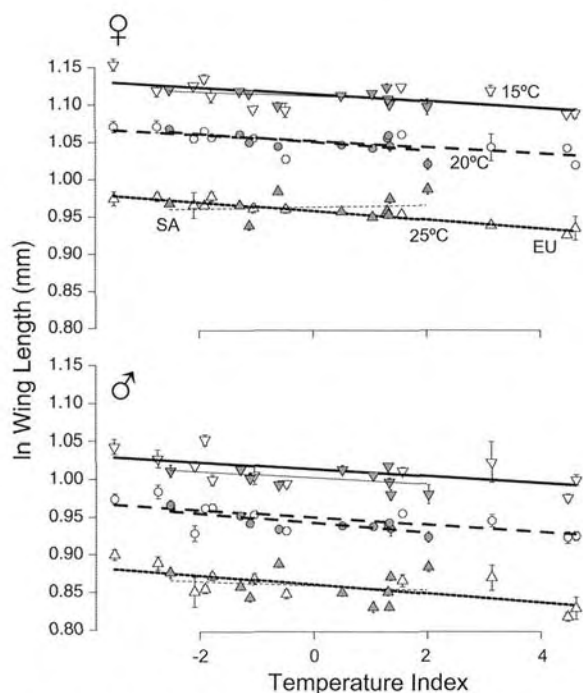


FIG. 3. Plastic and clinal variation in wing length. European flies are indicated by open symbols whereas South American flies are indicated by shaded symbols. Note that the T_{clime} index in SA has a much narrower range than in Europe. T_{dev} is indicated by the temperatures at the right.

TABLE 2. Regression statistics (mean \pm standard error) of various morphological variables (all ln transformed) on the Temperature Index.*

| Cont | Sex | T_{dev} | Wing Length | | Wing Loading | | Wet Weight | | Wing Area | |
|------|-----|-----------|------------------|-------------------------------|------------------|----------------------------|------------------|-----------------------------|------------------|-------------------------------|
| | | | Intercept | Slope | Intercept | Slope | Intercept | Slope | Intercept | Slope |
| EU | F | 15 | 1.11 \pm 0.005 | -0.005 \pm 0.0019* | 6.07 \pm 0.020 | 0.017 \pm 0.0073* | 7.48 \pm 0.025 | 0.006 \pm 0.0088 | 1.41 \pm 0.010 | -0.010 \pm 0.0035* |
| SA | F | 15 | 1.11 \pm 0.003 | -0.003 \pm 0.0020 | 6.12 \pm 0.020 | -0.017 \pm 0.0141 | 7.48 \pm 0.024 | -0.023 \pm 0.0170 | 1.36 \pm 0.007 | -0.006 \pm 0.0050 |
| EU | M | 15 | 1.01 \pm 0.006 | -0.005 \pm 0.0020+ | 5.93 \pm 0.020 | 0.018 \pm 0.0073* | 7.14 \pm 0.023 | 0.007 \pm 0.0081 | 1.22 \pm 0.012 | -0.011 \pm 0.0041* |
| SA | M | 15 | 1.00 \pm 0.004 | -0.004 \pm 0.0029 | 6.00 \pm 0.012 | -0.009 \pm 0.0082 | 7.14 \pm 0.020 | -0.019 \pm 0.0138 | 1.15 \pm 0.010 | -0.010 \pm 0.0069 |
| EU | F | 20 | 1.05 \pm 0.004 | -0.004 \pm 0.0013* | 6.14 \pm 0.020 | -0.001 \pm 0.0073 | 7.43 \pm 0.023 | -0.011 \pm 0.0082 | 1.29 \pm 0.007 | -0.009 \pm 0.0025** |
| SA | F | 20 | 1.05 \pm 0.004 | -0.006 \pm 0.0025+ | 6.18 \pm 0.013 | -0.014 \pm 0.0089 | 7.42 \pm 0.015 | -0.027 \pm 0.0102* | 1.24 \pm 0.007 | -0.013 \pm 0.0051* |
| EU | M | 20 | 0.95 \pm 0.005 | -0.005 \pm 0.0017** | 5.98 \pm 0.030 | -0.010 \pm 0.0105 | 7.06 \pm 0.034 | -0.021 \pm 0.0120 | 1.08 \pm 0.008 | -0.011 \pm 0.0028** |
| SA | M | 20 | 0.94 \pm 0.002 | -0.006 \pm 0.0017** | 6.06 \pm 0.020 | -0.004 \pm 0.0142 | 7.09 \pm 0.021 | -0.015 \pm 0.0147 | 1.03 \pm 0.005 | -0.011 \pm 0.0034* |
| EU | F | 25 | 0.96 \pm 0.002 | -0.006 \pm 0.0005*** | 6.20 \pm 0.022 | 0.009 \pm 0.0078 | 7.30 \pm 0.025 | -0.001 \pm 0.0089 | 1.10 \pm 0.008 | -0.010 \pm 0.0028** |
| SA | F | 25 | 0.96 \pm 0.005 | 0.001 \pm 0.0036 | 6.21 \pm 0.025 | -0.022 \pm 0.0175 | 7.27 \pm 0.019 | -0.020 \pm 0.0131 | 1.06 \pm 0.011 | 0.002 \pm 0.0073 |
| EU | M | 25 | 0.86 \pm 0.005 | -0.006 \pm 0.0019* | 6.06 \pm 0.022 | 0.010 \pm 0.0078 | 6.96 \pm 0.021 | -0.005 \pm 0.0073 | 0.90 \pm 0.007 | -0.015 \pm 0.0025*** |
| SA | M | 25 | 0.86 \pm 0.007 | -0.003 \pm 0.0048 | 6.09 \pm 0.020 | -0.017 \pm 0.0139 | 6.94 \pm 0.018 | -0.022 \pm 0.0124 | 0.85 \pm 0.013 | -0.005 \pm 0.0088 |

* All regressions were carried out on the population means within each continent (EU = Europe, SA = South America) of males and females at the specified T_{dev} . Significant slopes indicating clinal patterns are presented in bold. All of the intercepts were significantly different from zero. Statistics testing various hypotheses for plastic and clinal patterns are presented in the text.

Significance levels: + = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

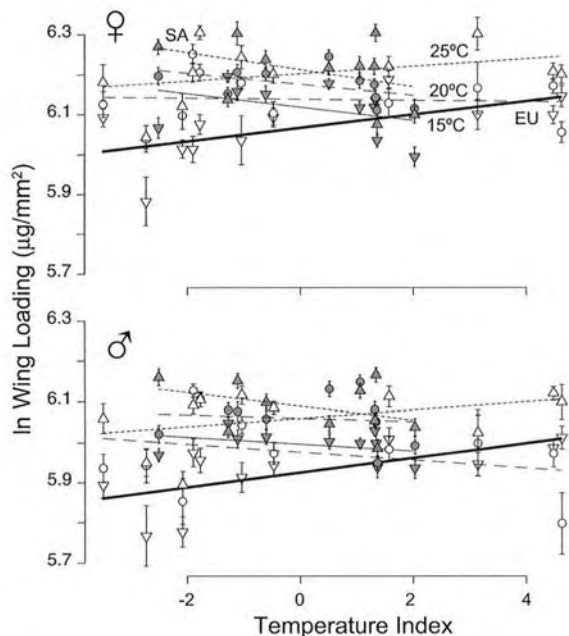


FIG. 4. Plastic and clinal variation in wing loading. European flies are indicated by circles whereas South American flies are indicated by inverted triangles. Note that the T_{cline} index in SA has a much narrower range than in Europe. T_{dev} is indicated by the temperatures at the right.

climates (Fig. 4, Table 2). SA flies exhibit the opposite: flies from cold sites have higher wing loadings. Interestingly, flies from either Europe or SA reared at the presumed temperature optimum for *D. subobscura* (20°C) show relatively little clinal pattern in wing loading (Table 2). Thus flies adapted to cooler climates tend to reduce wing loading more when reared at cool temperatures. In contrast, flies adapted to warm climate that develop at warm temperatures show less of a response. Moreover, this plastically mediated pattern of adaptation is present only in the ancestral European population, and not in the South American invaders. Although the European sites which we studied span a broader T_{cline} range than the SA sites, restricting the analysis to populations spanning only the SA T_{cline} range yields similar results to the full dataset.

Patterns of variation in the components of wing loading

We examined clinal variation in wet weight and in total wing area to determine which (or both) contributed to the clinal patterns of wing loading. Significant plastic variation in wet weight was apparent, showing a significant linear decline with T_{dev} (linear coefficient: -0.1357 , $F_{[1, 117]} = 156.99$, $P < 0.0001$) and no significant differences in this pattern between the continents ($F_{[1, 117]} = 0.418$, $P > 0.5$). Clinal variation in wet weight was very weak: most slopes declined with T_{cline} , but with slopes not significantly different from zero (Table 2). Nonetheless, there was a significant interaction between T_{cline} and continent ($F_{[1, 1117]} =$

5.56 , $P = 0.02$), with Old World populations showing a weak positive slope in flies reared at 15°C.

Wing area showed highly significant plastic increases at low T_{dev} (linear coefficient: -0.2174 , $F_{[1, 120]} = 2551.71$, $P < 0.0001$) and no significant interactions with Sex or Continent (all $P > 0.1$). In contrast to the weak clinal pattern in wet weight, wing area showed strong negative slopes with T_{cline} at all rearing temperatures (Table 2). Although no significant interaction between Continent and T_{cline} was apparent ($F_{[1, 119]} = 2.016$, $P > 0.15$), clinal patterns appear somewhat steeper at 25°C in Europe than in South America (Table 2). Moreover, wing area in the ancestral European flies was significantly greater overall than in the SA invaders ($F_{[1, 117]} = 111.40$, $P < 0.0001$).

DISCUSSION

In this paper, we raise the question of whether wing size clines might reflect selection imposed by the temperature sensitivity of flight. In insects, including *Drosophila*, cold temperatures lower wing beat frequency and thus power output (Reed *et al.*, 1942; Unwin and Corbet, 1984; Stevenson and Josephson, 1990), resulting in reduced flight performance. If flight is important for fitness, then reduced power output should favor reduced wing loading, the ratio of body mass to wing area, as this would help compensate for reduced flight performance (Reed *et al.*, 1942; Stalker, 1980; Starmer and Wolf, 1989; David *et al.*, 1994; Azevedo *et al.*, 1998). Reduced wing loading can be achieved by reducing body mass, by increasing wing area, or by both (Berrigan, 1991a; Dudley, 2000).

Opposing this adaptive expectation is a simple null hypothesis for the relationship between wing loading and environmental temperature. From dimensional analysis, wing loading should increase in proportion with linear body measures: this pattern is consistently observed in empirical comparisons among species (Bartholomew and Heinrich, 1973; Casey and Joos, 1983; Starmer and Wolf, 1989; Dudley, 2000). Thus, we have a qualitative contrast between predicted patterns of adaptive *versus* nonadaptive changes in wing loading with temperature (Fig. 1).

Most comparative studies have not measured wing loading directly (*i.e.*, from estimates of both body mass and wing area). Instead, most use a linear measure of thorax size as an index of body mass (Starmer and Wolf, 1989; David *et al.*, 1994; Petavy *et al.*, 1997; Azevedo *et al.*, 1998; Karan *et al.*, 1998; Karan *et al.*, 2000). This approach, however, may be misleading. Females typically have much larger abdomens than males, sometimes carrying egg loads that exceed 100% of their nonreproductive mass. In spite of the mass difference, sexual dimorphism in thorax length may be relatively small (Berrigan, 1991b). Thorax length may be more closely related to the total power output than to the total load that is being lifted (Marden, 1989a, b, 1995), thus it might be adaptive to have a relatively large thorax in cold temperatures.

The recent introduction of European *Drosophila*

subobscura into South America in the late 1970s (Brncic and Budnik, 1980; Brncic *et al.*, 1981) creates a “natural experiment” for testing hypotheses about adaptive vs. non-adaptive evolution. Latitudinal clines of the chromosomal arrangements began forming almost instantaneously in the South American invaders (Prevosti *et al.*, 1988; Ayala *et al.*, 1989; Prevosti *et al.*, 1990; Balanyà *et al.*, 2003). In contrast, wing size clines were not evident in SA (Budnik *et al.*, 1991; Pegueroles *et al.*, 1995) about one decade after the introduction (1986). Two decades after the introduction, however, a significant wing size cline for SA females has evolved and is essentially parallel to the ancestral cline in Europe (Fig. 2 and Calboli *et al.*, 2003; Gilchrist *et al.*, 2004). Most evidence suggests that the New World colonists are derived from the Mediterranean region of southern Europe (Prevosti *et al.*, 1987, 1988, 1989; Ayala *et al.*, 1989; Mestres and Serra, 1995; Mestres and Pascual, in preparation). If so, then the original colonists should have had a relatively small wing size and a large wing loading. Furthermore, those colonists should have maintained ancestral patterns of plasticity, adaptive or otherwise, barring some significant change in genetic architecture associated with a genetic bottleneck. Thus, both the ancestral and colonizing populations should show similar patterns of plasticity in wing size and wing loading in response to T_{dev} .

If selection on wing size is partially for enhanced flight in the cold, with a correlated reduction in wing loading, then we would expect to see a qualitatively similar pattern of decreasing wing size and increasing wing loading in response to T_{cline} on both continents. Of course, the high-latitude South American flies have not had long to adapt to cold conditions, and so might still show relatively high wing loading, compared to high-latitude flies in Europe.

Plastic and clinal variation in wing length

Ancestral and introduced populations of *D. subobscura* show patterns of both plastic and clinal variation in wing length (Fig. 3). Plastic variation in wing length is negatively related to T_{dev} , as expected. This is consistent with previous studies of plastic variation in wing and body size in a variety of ectotherms (reviewed in Atkinson, 1994; Partridge and French, 1996).

Genetic variation in wing length is negatively correlated with T_{cline} (Fig. 3, Table 2). Replicated wing size clines represent one of the most often cited signals of natural selection at work (Endler, 1977). Most native and introduced drosophilids show latitudinal variation in wing size, with similar patterns occurring in species on different continents (*D. pseudoobscura*: [Sokoloff, 1965]; *D. robusta*: [Stalker and Carson, 1947]; *D. simulans*: [David and Bocquet, 1975]; *D. obscura*: [Pegueroles *et al.*, 1995]; *D. melanogaster*: [Coyne and Beecham, 1987; Capy *et al.*, 1993; Imasheva *et al.*, 1994; James *et al.*, 1995; van't Land *et*

al., 1995; Gilchrist *et al.*, 2000]); *Zaprionus indianus*: [Karan *et al.*, 2000]).

Although our flies show thermal/latitudinal clines in size, we were surprised to find relatively flat slopes of wing size on T_{cline} in both females and males in the SA colonists at 25°C (Fig. 3, Table 2). Whereas T_{dev} has no effect on the slopes of the European clines, this is clearly not the case for SA flies. Further research into the significance of this pattern is currently underway.

Plasticity and clinal variation in wing loading

The observed plasticity in wing loading is consistent with the adaptive hypothesis in both European and South American *D. subobscura* (Fig. 1). Flies reared at lower T_{dev} have lower wing loadings than flies reared at higher T_{dev} (Fig. 4). This is true of both sexes and both continents, as one would expect if the New World colonists brought the genetic basis for this trait with them. What accounts for the decreased wing loading at low T_{dev} ? Wet weight increases with decreasing T_{dev} , however, wing area also increases and does so dramatically (Table 2). Thus, both ancestral and invading flies developing at low T_{dev} achieve reduced wing loading despite having larger masses.

In contrast, the genetically based changes in wing loading across patterns of T_{cline} (Fig. 4) show a mixture of similarities and differences in pattern between the ancestral and invading continents. First, wing loading in either females or males reared at 20°C shows no significant cline on either continent, an ambiguous result with regard to our hypotheses. But at rearing temperatures of 15 and 25°C, a sharp difference emerges between continents. Clinal slopes for the ancestral European females and males at these T_{dev} 's are all positive, whereas those for their South American counterparts are all negative. Thus at T_{dev} of 15 and 25°C, the European flies show a pattern consistent with adaptive clinal variation in wing loading, whereas the South American flies show a nonadaptive pattern of variation consistent with our null hypothesis. The adaptive clinal pattern is strongest at T_{dev} of 15°C, where the European flies of both sexes have evolved significantly lower wing loadings in cooler, high-latitude populations than are found in warmer, low latitude populations.

The results presented here are generally consistent with the previous studies combining plastic and clinal variation in wing loading in drosophilids. Azevedo and colleagues (1998) studied clinal and plastic variation in 22 populations of Australian *D. melanogaster*. These populations had already been shown to genetically increase in wing and thorax length with increasing latitude and decreasing temperature (James *et al.*, 1995). Azevedo *et al.* (1998) used the ratio of (wing area)^{0.5}/(thorax length) to yield a null expectation of zero slope for the regression of this wing-loading index on latitude [Note: because of a typographical error, the square root in this formula is missing throughout the paper: R. Azevedo, personal communication]. They found that the square root of wing area increased more rapidly than thorax length across latitudes. They

therefore rejected the null hypothesis in favor of the adaptive clinal expectation. Likewise, they found that wing loading increased with T_{dev} , supporting the expected adaptive pattern of plasticity. Similarly, Morin *et al.* (1999) examined plastic and genetic variation in French and Caribbean populations of *D. melanogaster* and *D. simulans*. In both species, flies reared at lower temperatures and from cooler habitats (France) had lower wing loadings despite having the generally larger wing and thorax sizes in flies reared in or adapted to the cold.

A few additional studies have examined indices of wing loading. Karan (1998, 2000) found a positive latitudinal cline in the wing length/thorax length ratio (inversely related to wing loading) in two species of drosophilids, a result consistent with the hypothesis of an adaptive response to clinal variation in temperature. David *et al.* (1994) and Petavy (1997) found an adaptive pattern of plasticity: larger wing/thorax length ratios were generally found in flies reared under cooler conditions.

Adaptive reductions in wing loading could be achieved either through a reduction in body mass or an increase in wing area (or both), however, the costs of these solutions may differ significantly. Increasing wing area is probably relatively inexpensive, in terms of material and energy. In contrast, reducing body mass extracts a significant cost because it comes at a cost of reproductive investment (Marden, 1989b, 1995; Berrigan, 1991b); female body mass in many insects is highly correlated with fecundity (Nunney and Cheung, 1997; Reeve and Fairbairn, 1999). In the ancestral European populations, wing area decreases more steeply and body mass decreases less steeply with T_{cline} than in SA, especially at 15°C.

The SA flies do not exhibit an adaptive clinal pattern of wing loading at any T_{dev} . Instead, wing loading increases positively with wing size and body mass (and negatively with T_{cline}), a pattern that must have evolved in the SA flies since their introduction in the late 1970s. This apparently maladaptive pattern (at least in the context of flight in cold weather), suggests that some factor other than flight performance has favored the evolution of clines where overall size simply decreases with temperature (Table 2). It remains unclear if this selection is imposed by a direct advantage of being large in the cold and/or small in the warm (*e.g.*, McCabe and Partridge, 1997; Reeve *et al.*, 2000), or if it might arise as a correlated response to selection on development time (Nunney, 1996; Partridge and French, 1996; Bochdanovits and de Jong, 2003; Santos *et al.*, 2004).

In contrast, the European flies show an apparently adaptive interaction between plastic (in response to T_{dev}) and genetic (in response to T_{cline}) adjustments in wing loading. In the ancestral European populations of *Drosophila subobscura*, there is little clinal variation in wing loading in flies reared under near-optimal rearing conditions of 20°C (Maynard-Smith, 1957; Maynard-Smith, 1958). Rearing under cool (15°C) condi-

tions reveals an adaptive, positive relationship between wing loading and the T_{cline} of the source population: cold-reared flies from cool climates decrease their wing loadings to a greater degree than do cold-reared flies from warm climates. This plastic adjustment of the clinal pattern would allow cold-adapted populations to maintain relatively high wing loadings (and, therefore, potentially higher fecundity) during parts of the year when the weather is warm and flight is less thermally constrained. Perhaps this represents a secondary phase of clinal evolution that eventually will take place in the South American invaders. Future selection experiments and studies of introduced flies in North America may help to better understand these patterns.

ACKNOWLEDGMENTS

This research has been supported by NSF grants DEB0242313 (GWG), DEB9981598 (RBH) DEB9629822 (RBH & GWG), and EF0328594 (GWG) and a Jeffress Research Grant (GWG). Comments from D. Berrigan, R. B. R. Azevedo, M. Angilletta, and two anonymous reviewers have improved this paper.

REFERENCES

- Atkinson, D. 1994. Temperature and organism size—a biological law for ectotherms? *Adv. Ecol. Res.* 25:1–58.
- Ayala, F. J., L. Serra, and A. Prevosti. 1989. A grand experiment in evolution: The *Drosophila subobscura* colonization of the Americas. *Genome* 31:246–255.
- Azevedo, R. B. R., A. C. James, J. McCabe, and L. Partridge. 1998. Latitudinal variation of wing: thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution* 52:1353–1362.
- Balanyà, J., L. Serra, G. W. Gilchrist, R. B. Huey, M. Pascual, F. Mestres, and E. Solé. 2003. Evolutionary pace of the chromosomal polymorphism of *D. subobscura* in colonizing populations. *Evolution* 57:1837–1845.
- Bartholomew, G. A. and B. Heinrich. 1973. A field study of flight temperatures in moths in relation to body weight and wing loading. *J. Exp. Biol.* 58:123–135.
- Beckenbach, A. T. and A. Prevosti. 1986. Colonization of North America by the European species, *Drosophila subobscura* and *D. ambigua*. *Am. Midl. Nat.* 115:10–18.
- Berrigan, D. 1991a. Lift production in the flesh fly *Neobellieria* (= *Sarcophaga*) *bullata*. *Func. Ecol.* 5:448–456.
- Berrigan, D. 1991b. The allometry of egg size and number in insects. *Oikos* 60:313–321.
- Bochdanovits, Z. and G. de Jong. 2003. Experimental evolution in *Drosophila melanogaster*: Interaction of temperature and food quality selection regimes. *Evolution* 57:1829–1836.
- Brncic, D. and M. Budnik. 1980. Colonization of *Drosophila subobscura* Collin in Chile. *Dros. Inf. Serv.* 55:20.
- Brncic, D., A. Prevosti, M. Budnik, M. Monclus, and J. Ocaña. 1981. Colonization of *Drosophila subobscura* in Chile. I. First population and cytogenetic studies. *Genetica* 56:3–9.
- Budnik, M., L. Cifuentes, and D. Brncic. 1991. Quantitative analysis of genetic differentiation among European and Chilean strains of *Drosophila subobscura*. *Heredity* 67:29–33.
- Calboli, F. C. F., G. W. Gilchrist, and L. Partridge. 2003. Different contribution of cell size and cell number in two newly established and one ancient body size clines of *Drosophila subobscura*. *Evolution* 57:566–573.
- Capy, P., E. Pla, and J. R. David. 1993. Phenotypic and genetic variability of morphological traits in natural populations of *Drosophila melanogaster* and *Drosophila simulans*. I. Geographic variations. *Genetics, Selection, Evolution* 25:517–536.

- Casey, T. M. and B. A. Joos. 1983. Morphometrics, conductance, thoracic temperature, and flight energetics of noctuid and geometrid moths. *Phys. Zool.* 56:160–173.
- Conover, D. O. and T. M. C. Present. 1990. Countergradient variation in growth rate: Compensation for length of the growing season among Atlantic silversides from different latitudes. *Oecologia* 83:316–324.
- Coyne, J. A. and E. Beecham. 1987. Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. *Genetics* 117:727–737.
- Curtsinger, J. W. and C. C. Laurie-Ahlberg. 1981. Genetic variability of flight metabolism in *Drosophila melanogaster*. I. Characterization of power output during tethered flight. *Genetics* 98:549–564.
- David, J. R. and C. Bocquet. 1975. Evolution in a cosmopolitan species: Genetic latitudinal clines in *Drosophila melanogaster* wild populations. *Experientia* 31:164–166.
- David, J. R., B. Moreteau, J.-P. Gauthier, G. Petavy, J. Stockel, and A. G. Imasheva. 1994. Reaction norms of size characters in relation to growth temperature in *Drosophila melanogaster*: An isofemale line analysis. *Gen. Sel. Evol.* 26:229–251.
- Dudley, R. 2000. *The biomechanics of insect flight: Form, function, evolution*. Princeton University Press, Princeton.
- Ellington, C. P. 1984. The aerodynamics of hovering insect flight. VI. Lift and power requirements. *Phil. Trans. R. Soc. London B* 305:145–181.
- Endler, J. A. 1977. *Geographic variation, speciation, and clines*. Princeton University Press, Princeton.
- Gilchrist, A. S., R. B. R. Azevedo, L. Partridge, and P. O'Higgins. 2000. Adaptation and constraint in the evolution of *Drosophila melanogaster* wing shape. *Evol. Devel.* 2:114–124.
- Gilchrist, G. W., R. B. Huey, J. Balanyà, M. Pascual, and L. Serra. 2004. A time series of evolution in action: Latitudinal cline in wing size in South American *Drosophila subobscura*. *Evolution* 58:768–780.
- Gilchrist, G. W., R. B. Huey, and L. Serra. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. *Genetica* 112–113:273–286.
- Huey, R. B., D. Berrigan, G. W. Gilchrist, and J. C. Herron. 1999. Testing the adaptive significance of acclimation: A strong inference approach. *Amer. Zool.* 39:135–148.
- Huey, R. B., G. W. Gilchrist, M. L. Carlson, D. Berrigan, and L. Serra. 2000. Rapid evolution of a geographic cline in size in an introduced fly. *Science* 287:308–309.
- Imasheva, A. G., O. A. Bubli, and O. E. Lazebny. 1994. Variation in wing length in Eurasian natural populations of *Drosophila melanogaster*. *Heredity* 72:508–514.
- James, A. C., R. B. R. Azevedo, and L. Partridge. 1995. Cellular basis and developmental timing in a size cline of *Drosophila melanogaster*. *Genetics* 140:659–666.
- Karan, D., A. K. Munjal, P. Gibert, B. Moreteau, R. Parkash, and J. R. David. 1998. Latitudinal clines for morphological traits in *Drosophila kikkawai*: A study of natural populations from the Indian subcontinent. *Gen. Res.* 71:31–38.
- Karan, D., S. Dubey, B. Moreteau, and R. Parkash. 2000. Geographical clines for quantitative traits in natural populations of a tropical drosophilid: *Zaprionus indianus*. *Genetica* 108:91–100.
- Ludwig, D. and L. Rowe. 1990. Life-history strategies for energy gain and predator avoidance under time constraints. *Am. Nat.* 135:686–707.
- Marden, J. H. 1989a. Bodybuilding dragonflies: Costs and benefits of maximizing flight muscle. *Phys. Zool.* 62:505–521.
- Marden, J. H. 1989b. Effects of load-lifting constraints on the mating system of a dance fly. *Ecology* 70:496–502.
- Marden, J. H. 1995. Evolutionary adaptation of contractile performance in muscle of ectothermic winter-flying moths. *J. Evol. Biol.* 198:2087–2094.
- Maynard-Smith, J. 1957. Temperature tolerance and acclimatization in *Drosophila subobscura*. *J. Exp. Biol.* 34:85–96.
- Maynard-Smith, J. 1958. The effects of temperature and of egg-laying on the longevity of *Drosophila subobscura*. *J. Exp. Biol.* 35:832–842.
- McCabe, J. and L. Partridge. 1997. An interaction between environmental temperature and genetic variation for body size for the fitness of adult female *Drosophila melanogaster*. *Evolution* 51:1164–1174.
- Mestres, F. and L. Serra. 1995. On the origin of the O5 chromosomal inversion in American populations of *Drosophila subobscura*. *Hereditas* 123:39–46.
- Misra, R. K. and E. C. R. Reeve. 1964. Clines in body dimensions in populations of *Drosophila subobscura*. *Gen. Res.* 5:240–256.
- Morin, J. P., B. Moreteau, G. Petavy, and J. R. David. 1999. Divergence of reaction norms of size characters between tropical and temperate populations of *Drosophila melanogaster* and *D. simulans*. *J. Evol. Biol.* 12:329–339.
- Nunney, L. 1996. The response to selection for fast larval development in *Drosophila melanogaster* and its effect on adult weight: An example of a fitness trade-off. *Evolution* 50:1193–1204.
- Nunney, L. and W. Cheung. 1997. The effect of temperature on body size and fecundity in female *Drosophila melanogaster*: Evidence for adaptive plasticity. *Evolution* 51:1529–1535.
- Partridge, L. and V. French. 1996. Thermal evolution of ectotherm body size: Why get big in the cold? In I. A. Johnston and A. F. Bennett (eds.), *Animals and temperature: Phenotypic and evolutionary adaptation*, pp. 265–292. Cambridge University Press, Cambridge, UK.
- Pegueroles, G., M. Papaceit, A. Quintana, A. Guillén, A. Prevosti, and L. Serra. 1995. An experimental study of evolution in progress: Clines for quantitative traits in colonizing and Palearctic populations of *Drosophila*. *Evol. Ecol.* 9:453–465.
- Petavy, G., J. P. Morin, B. Moreteau, and J. R. David. 1997. Growth temperature and phenotypic plasticity in two *Drosophila* sibling species: Probable adaptive changes in flight capacities. *J. Evol. Biol.* 10:875–887.
- Prevosti, A. 1955. Geographic variability in quantitative traits in populations of *Drosophila subobscura*. *Cold Spr. Har. Symp. Quant. Biol.* 20:294–298.
- Prevosti, A., G. Ribo, L. Serra, M. Aguade, J. Balanyà, M. Monclus, and F. Mestres. 1988. Colonization of America by *Drosophila subobscura*: Experiment in natural populations that supports the adaptive role of chromosomal-inversion polymorphism. *Proc. Natl. Acad. Sci. U.S.A.* 85:5597–5600.
- Prevosti, A., L. Serra, M. Aguade, G. Ribo, F. Mestres, and J. Balanyà. 1989. Colonization and establishment of the Palearctic species *Drosophila subobscura* in North and South America. In A. Fontdevila (ed.), *Evolutionary biology of transient and unstable populations*, pp. 114–129. Springer Verlag, Berlin.
- Prevosti, A., L. Serra, M. Monclus, F. Mestres, A. LaTorre, G. Ribo, and M. Aguade. 1987. Colonización de América por *Drosophila subobscura*. *Evolucion Biologica* 1:1–24.
- Prevosti, A., L. Serra, C. Segarra, M. Aguade, G. Ribo, and M. Monclus. 1990. Clines of chromosomal arrangements of *Drosophila subobscura* in South America evolve closer to Old World patterns. *Evolution* 44:218–221.
- Reed, S. C., C. M. Williams, and L. E. Chadwick. 1942. Frequency of wingbeat as a character for separating species races and geographic varieties of *Drosophila*. *Genetics* 27:349–361.
- Reeve, J. P. and D. J. Fairbairn. 1999. Change in sexual size dimorphism as a correlated response to selection on fecundity. *Heredity* 83:697–706.
- Reeve, M. W., K. Fowler, and L. Partridge. 2000. Increased body size confers greater fitness at lower experimental temperature in male *Drosophila melanogaster*. *J. Evol. Biol.* 13:836–844.
- Roff, D. 1981. On being the right size. *Am. Nat.* 118:405–422.
- Santos, M., P. J. F. Iriarte, W. Céspedes, J. Balanyà, A. Fontdevila, and L. Serra. 2004. Swift laboratory thermal evolution of wing shape (but not size) in *Drosophila subobscura* and its relationship with chromosomal inversion polymorphism. *J. Evol. Biol.* 17:841–855.
- Sibley, R. M. and D. Atkinson. 1994. How rearing temperature affects optimal adult size in ectotherms. *Func. Ecol.* 8:486–493.
- Sokoloff, A. 1965. Geographic variation of quantitative characters in populations of *Drosophila pseudoobscura*. *Evolution* 19:300–310.
- Stalker, H. D. 1980. Chromosome studies in wild populations of

- Drosophila melanogaster*. II. Relationship of inversion frequencies to latitude, season, wing-loading and flight activity. *Genetics* 95:211–223.
- Stalker, H. D. and H. L. Carson. 1947. Morphological variation in natural populations of *Drosophila robusta* Sturtevant. *Evolution* 1:237–248.
- Starmer, W. T. and L. L. Wolf. 1989. Causes of variation in wing loading among *Drosophila* species. *Biol. J. Linn. Soc.* 37:247–261.
- Stevenson, R. D. and R. K. Josephson. 1990. Effects of operating frequency and temperature on mechanical power output from moth flight muscle. *J. Exp. Biol.* 198:61–78.
- Unwin, D. M. and S. A. Corbet. 1984. Wingbeat frequency, temperature, and body size in bees and flies. *Phys. Entomol.* 9:115–121.
- van der Have, T. M. and G. de Jong. 1996. Adult size in ectotherms: Temperature effects on growth and differentiation. *J. Theoret. Biol.* 183:329–340.
- Van Voorhies, W. A. 1996. Bergmann size clines: A simple explanation for their occurrence in ectotherms. *Evolution* 50:1259–1264.
- van't Land, J., P. van Putten, H. Villarroel, A. Kamping, and W. van Delden. 1995. Latitudinal variation in wing length and allele frequencies for *Adh* and α -*Gpdh* in populations of *Drosophila melanogaster* from Ecuador and Chile. *Dros. Inf. Serv.* 76:156.
- von Bertalanffy, L. 1960. Principles and theory of growth. In W. W. Nowinskii (ed.), *Fundamental aspects of normal and malignant growth*, pp. 137–259. Elsevier, Amsterdam.
- Williams, G. C. 1966. *Adaptation and natural selection*. Princeton University Press, Princeton, New Jersey.