

# Correlated responses to selection on body size in *Drosophila melanogaster*

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## Summary

Correlated responses to artificial selection on body size in *Drosophila melanogaster* were investigated, to determine how the changes in size were produced during development. Selection for increased thorax length was associated with an increase in larval development time, an extended growth period, no change in growth rate, and an increased critical larval weight for pupariation. Selection for reduced thorax length was associated with reduced growth rate, no change in duration of larval development and a reduced critical larval weight for pupariation. In both lines selected for thorax length and lines selected for wing area, total body size changed in the same direction as the artificially selected trait. In large selection lines of both types, the increase in size was achieved almost entirely by an increase in cell number, while in the small lines the decrease in size was achieved predominantly by reduced cell size, and also by a reduction in cell number. The implications of the results for evolutionary-genetic change in body size in nature are discussed.

## 1. Introduction

The size of an organism has important effects on its physiology, life history and ecology (e.g. Bonner, 1965; Peters, 1983; Calder, 1984; Schmidt-Neilsen, 1984; Damuth, 1987) and, in most animals, a characteristic adult body size is achieved by the control of growth during embryonic and post-embryonic stages. It is of great interest to understand the processes of growth control and the ways in which they may be modified to produce a change in body size during evolution. In the present study, using the fruit fly *Drosophila melanogaster*, we have artificially selected for large and small adult body size, to determine how different aspects of growth control contribute to the changes in size. Insect size is determined by the dimensions of the surface epidermis, and we have established whether the size changes that occur in

response to selection are attributable to alterations in epidermal cell number, cell size or both.

In *Drosophila*, the adult head and thorax are formed by discrete epidermal imaginal discs that grow inside the larva and then fuse together and replace the larval epidermis at metamorphosis. The two wing discs, for example, each come from about 20 cells in the hatchling larva, grow to around 50 000 cells by the late third instar (Bryant & Levinson, 1985), evaginate and then undergo further cell divisions after pupariation, fuse with adjacent thoracic discs and finally secrete the adult cuticle of the dorsal mesothorax and the wings.

Early in the third (and final) larval instar, hormonal events are initiated that lead to pupariation. This is associated with the larva reaching a 'critical weight' and larvae starved below that weight fail to pupariate, despite surviving past the time at which this normally occurs (Bakker, 1959; Robertson, 1963; Partridge *et al.*, 1994*b*). The minimum size of an adult is therefore set by the larval size at which the decision is taken to pupariate. After that critical point there is normally a fixed period of feeding and then the larva wanders

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from the food, empties the gut and pupariates. Restricted feeding in the post-critical period has little effect on pupariation time but produces an adult of reduced size (Bakker, 1959; Robertson, 1963), with decreases in both cell number and cell size (Simpson, 1979; Held, 1979).

There is evidence, however, that body size of the adult fly is not determined simply by food acquisition during the post-critical period, because the imaginal discs display targeted growth to a fixed size. Growth termination appears to be largely intrinsic to the imaginal discs, given full nutrition (Bryant & Simpson, 1984). Hence, early damage to some discs will delay pupariation, but no part of the resulting fly becomes abnormally large, indicating that the undamaged discs do not continue growing beyond their normal size (Simpson *et al.*, 1980). Furthermore, young imaginal wing discs transplanted into the adult abdomen continue to grow normally, but only up to approximately the normal cell number, despite permissive conditions for further growth (Bryant & Levinson, 1985). This targeted growth will determine the maximum number of cells that the adult structure can contain, but much less is understood of factors controlling the size of the cells.

Natural populations of *Drosophila* harbour abundant genetic variation for body size, with the trait showing a narrow-sense heritability of between 0.2 and 0.6 in the laboratory (Robertson & Reeve, 1952; Reeve & Robertson, 1953) and 0.2–0.3 in nature (Coyne & Beecham, 1987; Riska *et al.*, 1989; Prout & Barker, 1989; van't Land *et al.*, 1998). Previous work has shown that lines artificially selected for large and small body size differ in pre-adult development time, with larger lines taking longer to develop than the small or control lines (Reeve, 1954; Sang, 1956; Robertson, 1957, 1960, 1963; Partridge & Fowler, 1993). Apart from these data, little is known of the changes during development that result in altered body size, nor whether this is attributable to changes in cell number, cell size or both. From the outline of size control given above, it follows that a greater body size would necessitate either an increase in the critical size for pupariation (to allow for greater total growth), or an increase in the rate or duration of growth in the post-critical period. In addition, the increased body size would have to result from either an increased target cell number, an increased cell size, or both. In contrast, small body size could result from reduced critical size for pupariation or from a reduction in the subsequent duration or rate of growth, and no adjustment to target cell number or cell size would be required.

In the present study we determined the role of these factors in producing the response to artificial selection for body size. For different selection lines we measured development time, growth rate, critical weight and

age for pupariation, wet weight of growth-terminated larvae, the size of the resulting adults, and the cell size and cell number in their wings.

## 2. Materials and methods

### (i) General methods

All experiments were carried out at 25 °C. Routine propagation of strains, and the measurement of development time and size traits on fully fed flies, were done using cultures maintained on Lewis medium: 13.76 g agar, 187.75 g sucrose, 205.75 g maize meal, 37.05 g flaked yeast and 60 ml nipagin solution in 2200 ml water. Eggs were collected on grape juice medium: 300 ml of grape juice concentrate, 40 g agar and 500 ml water, with a small piece of yeast paste on the surface. For measuring larval growth rates and critical weights for pupariation (and also size traits on starved flies), larvae were grown in small chambers in yeast suspension over agar: the agar was made up at 15 g/l, and the yeast was made up at 20 g in 35 ml water. Water was added as necessary to maintain a concentrated but non-sticky yeast suspension.

### (ii) Selection lines

Most of the experimental work was done with lines artificially selected for thorax length. Also, the information on correlated changes in size in other parts of the fly and on the cellular basis of size change, was supplemented with data from a separate set of lines artificially selected for wing area.

The lines selected for thorax length have been described previously (Partridge & Fowler, 1993; Santos *et al.*, 1992, 1994). They were derived from a random-bred stock collected in Dahomey (now Benin, West Africa) in 1970, and maintained since in population cage culture. Four replicate lines were selected for large or for small thorax length, with four replicate control lines. Thorax length (strictly, the length of the dorsal mesothorax) was measured under a dissecting microscope fitted with an eyepiece graticule. The fly was laid on its side, and the distance from the base of the most anterior macrochaete to the top of the scutellum was noted. In each generation, 10 pairs of flies out of 25 measured were used as breeders in each line: the 10 with the largest thorax lengths for the LARGE lines and the 10 smallest for the SMALL lines. CONTROL lines, bred from 10 pairs picked at random, were included to accommodate evolutionary changes not attributable to selection itself. The lines were selected in this way for 55 generations, and then maintained without selection but at the same population size and with the same culture methods as those during selection. Selection was reinstated for two

generations before the experiments reported here were done and, by the time of the work with the  $1 \times 2$  and  $3 \times 4$  crosses, the lines had been in existence for 102 and 109 generations, respectively.

The selection lines had been maintained at small population size for many generations so, in all experiments, we worked with F1 hybrids between the lines within each selection regime, in order to abolish any effects of inbreeding depression on fitness-related traits (e.g. growth rate, development time and survival during starvation). The hybrids were made by crossing females of line 1 with males of line 2, and females of line 3 with males of line 4, using the arbitrary line number labels that had been assigned at the start of selection. The crosses were made using approximately 1500 adults of each line contributing to the cross. These hybrid 'thorax' lines were scored for pre-adult development time, larval growth rate and critical weight for pupariation, and for the thorax length, wing area and wing cell area of fully fed adults. The experiments with the  $1 \times 2$  and  $3 \times 4$  hybrids were performed in separate experimental blocks, for logistical reasons.

The lines selected for wing area were not replicated, and were derived from a base stock made from 48 isofemale lines collected in North Carolina in summer 1994. We used the males and females from each isofemale line once as parents in a circular crossing design. The offspring of these parents were grown up at low density (350 eggs per bottle), and were the first generation selected for wing area. The left wing from each fly was detached, mounted on a microscope slide and, using a camera lucida attachment to a binocular microscope, the area was traced onto a digitizing pad and calculated by computer. In the selection lines, the 40 males and females with the greatest (or smallest) wing area out of 100 flies of each sex measured, were used as parents of the next generation. A control line was maintained by taking 40 pairs at random in each generation. Selection was continued for 27 generations.

#### (iii) *Measurement of pre-adult development times*

Measurements of development time were made only on the 'thorax' lines. We measured both egg-to-pupariation time and the pupal period (strictly, the interval between pupariation and eclosion). A random sample of eggs from the 3000 parents was collected on grape juice medium from the two crosses within each selection regime and, from each cross, the newly hatched larvae were transferred in batches of 50 into each of 20 vials (75 mm  $\times$  25 mm diameter) containing 7 ml of Lewis medium with a drop of yeast suspension on the surface. The vials were scored every 8 h, and the position of each new puparium (defined by the

eclosion of the anterior spiracles) was marked on the outside of the vial. The vials were also scored every 8 h for the presence of adults, which were collected and sexed. These data were used to calculate egg-to-puparium period for the two sexes combined (they cannot be sexed at pupariation), and pupal period for the two sexes separately.

#### (iv) *Sizes of fully fed adults*

The 'thorax' line adults from the development time experiment (described above) were used to determine the thorax length, wing area and wing cell area of fully fed adults. Three females and three males were picked at random from all the eclosing adults from each vial, and were scored for thorax length (as in the production of the selection lines). Two different females and two different males from each vial were taken randomly and used for wing area measurements (using the method described above for artificial selection on wing area). Each cell in the wing blade secretes a single hair, or trichome (Dobzhansky, 1929). Cell density was measured by counting trichome density in three standard regions of both the dorsal and ventral wing surfaces (Fig. 1), on the wings used for area determination. The wings were examined under a compound microscope at  $\times 400$ . All trichomes in a 0.01 mm<sup>2</sup> area on each surface in each wing region were traced onto paper using a camera lucida, and the values for the three different regions were averaged to obtain an index of overall cell density and area on each wing surface.

The 'wing' selection lines were scored for wing area, cell area, body weight and thorax length. After setting up the selection bottles, the selected parental flies from generation 26 were allowed to lay extra eggs, which were picked at 50 eggs per vial for a total of 10 vials per line. The traits were measured on the same five individuals per sex per vial. Cell area was scored in one region of the dorsal surface of the wing (Fig. 1). Wet weights were measured (to the nearest 0.002 mg) on a Sartorius M500p microbalance, using flies 6 days after eclosion, to allow the females to reach a steady weight.

#### (v) *Growth curves*

Larval growth rates were examined only on the lines selected for thorax length. From each cross, eggs were collected on grape juice medium, and first instar larvae were collected after 24 h and counted in batches of 100 into yeast suspension over agar, as described above. Starting at 24 h and ending at 120 h post-egg lay, batches of 50 larvae taken from at least two different growth chambers were weighed every 12 h.

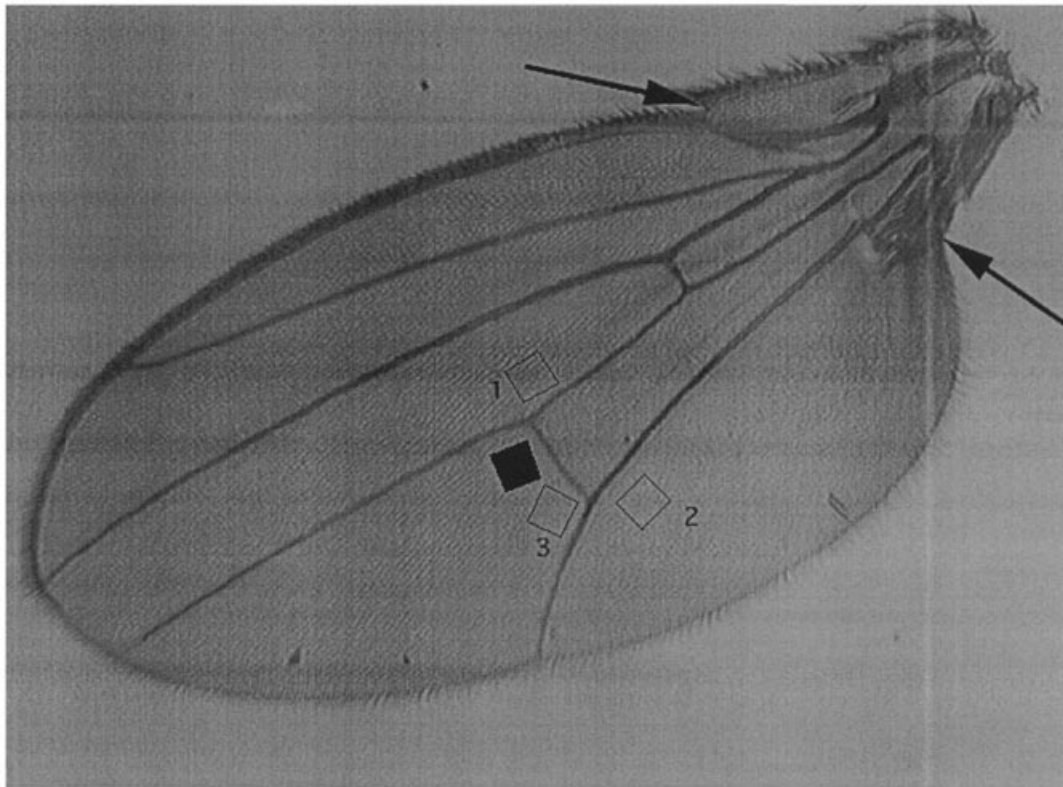


Fig. 1. The wing of *D. melanogaster*, showing the limits used for wing area measurement (arrows) and the standard regions used for trichome counts to estimate cell area in the 'thorax' selection lines (open squares 1–3) and in the 'wing' selection lines (filled square). On wings of different sizes, the regions were chosen by eye to correspond to the equivalent locations with respect to veins and the wing margin.

They were removed from the yeast paste, washed with insect Ringer (37.4 g NaCl, 1.75 g KCl, 1.30 g CaCl<sub>2</sub>, 0.50 g Na<sub>2</sub>HPO<sub>4</sub>, 0.25 g KH<sub>2</sub>PO<sub>4</sub>, made up to 5 L in distilled water) and dried gently on paper towels. Larvae were weighed to the nearest 0.002 mg on a Sartorius M500p microbalance and then placed individually in vials (51 mm × 12 mm diameter) containing yeast paste applied to damp tissue paper, to complete their development. This allowed the sex of the eclosing adults to be determined, and hence the construction of separate growth curves for male and female larvae.

#### (vi) Critical point for pupariation

The critical larval age and weight for pupariation were investigated only for the 'thorax' selection lines. Newly hatched first instar larvae were counted out into growth chambers and later they were removed from the yeast, washed with insect Ringer and weighed to the nearest 0.002 mg. Larvae were placed individually in vials, as described for the measurement of growth curves, except this time no yeast paste was provided. Only if the larvae were past their critical

age/weight could they pupariate without further feeding and, for each starved larva, we scored pupariation and eclosion. Sample sizes of larvae starved at each time point varied between 150 and 194. For the first set of hybrid LARGE, CONTROL and SMALL lines, larvae were starved at 72 h, 76 h and 80 h post-egg lay. For the second set of hybrid lines, larvae were starved at 72 h, 76 h, 80 h and 84 h post-egg lay. When starved larvae successfully completed development into scorable adults, these were sexed and measured for thorax length, in order to calculate the conversion efficiency of larval weight into adult size.

#### (vii) Statistical analysis

For development times and thorax lengths of the hybrid 'thorax' selection lines, the vial means were checked for normality (Shapiro–Wilk *W*-test). Data from only two of the 18 development times (six hybrid lines × three development time traits) were significantly ( $P < 0.05$ ) non-normal, and the data were not transformed for analysis. The experiments for the two sets of hybrid lines were analysed separately, because

Table 1. Development times and size traits of lines selected for thorax length

Size selection:	Small		Control		Large	
	1 × 2 114.6 (0.55)	3 × 4 118.7 (0.91)	1 × 2 114.7 (0.51)	3 × 4 118.4 (0.52)	1 × 2 120.3 (0.64)	3 × 4 127.5 (0.68)
Sex:	Female	Male	Female	Male	Female	Male
Pupal period (h)	89.6 (0.54)	97.8 (0.88)	88.3 (0.56)	92.0 (0.78)	89.4 (0.72)	96.7 (0.80)
Thorax length (mm)	0.985 (0.005)	0.845 (0.008)	1.079 (0.011)	0.937 (0.007)	1.161 (0.007)	1.109 (0.007)
Wing area (mm <sup>2</sup> )	1.63 (0.016)	1.23 (0.012)	1.81 (0.029)	1.39 (0.014)	1.96 (0.016)	1.85 (0.025)
Cell area (mm <sup>2</sup> × 10 <sup>-4</sup> )						
Dorsal	1.56 (0.018)	1.34 (0.008)	1.71 (0.014)	1.48 (0.012)	1.69 (0.019)	1.68 (0.018)
Ventral	1.78 (0.029)	1.51 (0.022)	1.93 (0.021)	1.67 (0.018)	1.91 (0.022)	1.86 (0.023)

Figures are the means (and 95% confidence limits) calculated from the vial means.

they were performed in separate experimental blocks. The vial means were subjected to one-way analysis of variance. *A posteriori* linear contrasts were used to determine the significance of deviation of the LARGE and SMALL hybrid lines from the CONTROLS.

The weight data for the larval growth measurements did not deviate from normality. For the two hybrid experiments separately, and for each time interval and sex, means and 95% confidence limits were calculated. When confidence limits of the selection lines overlapped with the controls, one-way analysis of variance with *a posteriori* linear contrasts was used to determine whether the weight differences were statistically significant.

For sizes of fully fed adults, none of the data for thorax length or wing area deviated significantly from normality ( $P < 0.05$ ). In the lines selected for thorax length, one of 24 distributions (two experiments, three selection line crosses, two sexes, dorsal and ventral counts) was significantly ( $P < 0.05$ ) non-normal. None of the data were transformed for analysis. To determine the contribution of cell area to the correlated response to selection in wing area in the thorax-length selection lines, one-way analysis of variance with contrasts between selection and control lines was done. To determine whether cell number also contributed to the response in the SMALL selection lines, the ratio of wing area to cell area in the selection lines was calculated, and used to produce an expected value for control wing area if cellular relations in the wing had been unaltered by selection. The expected value was then compared with the observed. The cellular basis of the response of wing area to selection in the lines selected for this trait was investigated in the same way.

The data from the starvation experiments, for determination of the critical point for pupariation, present some difficulties for analysis. First, there is a correlation between the two variables, larval age and weight at starvation, because the older larvae are, on average, larger. Secondly, because the SMALL lines grew more slowly, while the LARGE lines continued the phase of rapid growth for longer, the weights of larvae from the different lines starved at the same age were, on average, different. Thirdly, it was clear from the data that, for a given weight, a larva was more likely to pupariate if it was older (see also Partridge *et al.*, 1994b). We therefore opted for a conservative, non-parametric analysis of comparable data for weight-age combinations. We allocated the starved larvae to 0.1 mg weight categories for this comparison, and subjected the numbers of larvae giving rise to, or not giving rise to, adults to chi-squared analysis. All data were analysed using JMP (SAS Institute, 1994).

### 3. Results

#### (i) Development times

The egg-to-pupariation and pupal development times for the 'thorax' selected lines are given in Table 1. For each set of hybrid crosses, there were significant differences in egg-to-pupariation time ( $F = 141.6$ , d.f. 2/57 for the  $1 \times 2$  cross;  $F = 225.4$ , d.f. 2/57 for the  $3 \times 4$  cross,  $P < 0.0001$  in each case). The SMALL lines did not differ significantly from the CONTROL lines ( $P > 0.05$ ), while the LARGE lines had a larval period that exceeded that of the CONTROL lines by 5–6 h ( $P < 0.0001$ ). Analysis of variance for the pupal periods showed that, for both the females and the males of both sets of hybrid lines, the overall differences were statistically significant ( $1 \times 2$  cross, female pupal period  $F = 6.1$ , d.f. 2/57,  $P < 0.01$ , male pupal period  $F = 65.8$ , d.f. 2/57,  $P < 0.0001$ ;  $3 \times 4$  cross, female pupal period  $F = 35.4$ , d.f. 2/57,  $P < 0.0001$ , male pupal period  $F = 115.7$ , d.f. 2/57,  $P < 0.0001$ ). Contrast analysis showed that, for both sexes in both sets of hybrid lines, the SMALL lines had longer pupal periods than the CONTROL lines ( $P < 0.001$  for all comparisons). For the LARGE lines the data were less consistent, with both sexes showing

longer pupal periods than the CONTROL lines for the  $1 \times 2$  cross (non-significant for females,  $P < 0.01$  for males) while, for the  $3 \times 4$  cross lines the LARGE lines had significantly ( $P < 0.0001$ ) shorter pupal periods than the CONTROL lines.

#### (ii) Larval growth

Growth curves were derived from weight measurements of larvae from the two sets of hybrid 'thorax' selection lines (Fig. 2). As usual in *Drosophila* (see Partridge *et al.*, 1994*b*), female larvae grew faster than males and achieved higher weights at maturity. Furthermore, in each sex the SMALL lines grew distinctly more slowly than either of the other lines. SMALL line larvae were first significantly ( $P < 0.05$ ) lighter than those from the CONTROL and LARGE lines at 48 h after egg-lay, and they deviated progressively thereafter. Larvae from the LARGE and CONTROL lines did not show any significant differences in weight before 96 h, but the LARGE lines continued growth for longer than the CONTROL lines before starting the growth-terminated wandering phase and then pupariating. The final, 120 h sample was taken after many of the larvae had already pupariated, and may therefore not be a random

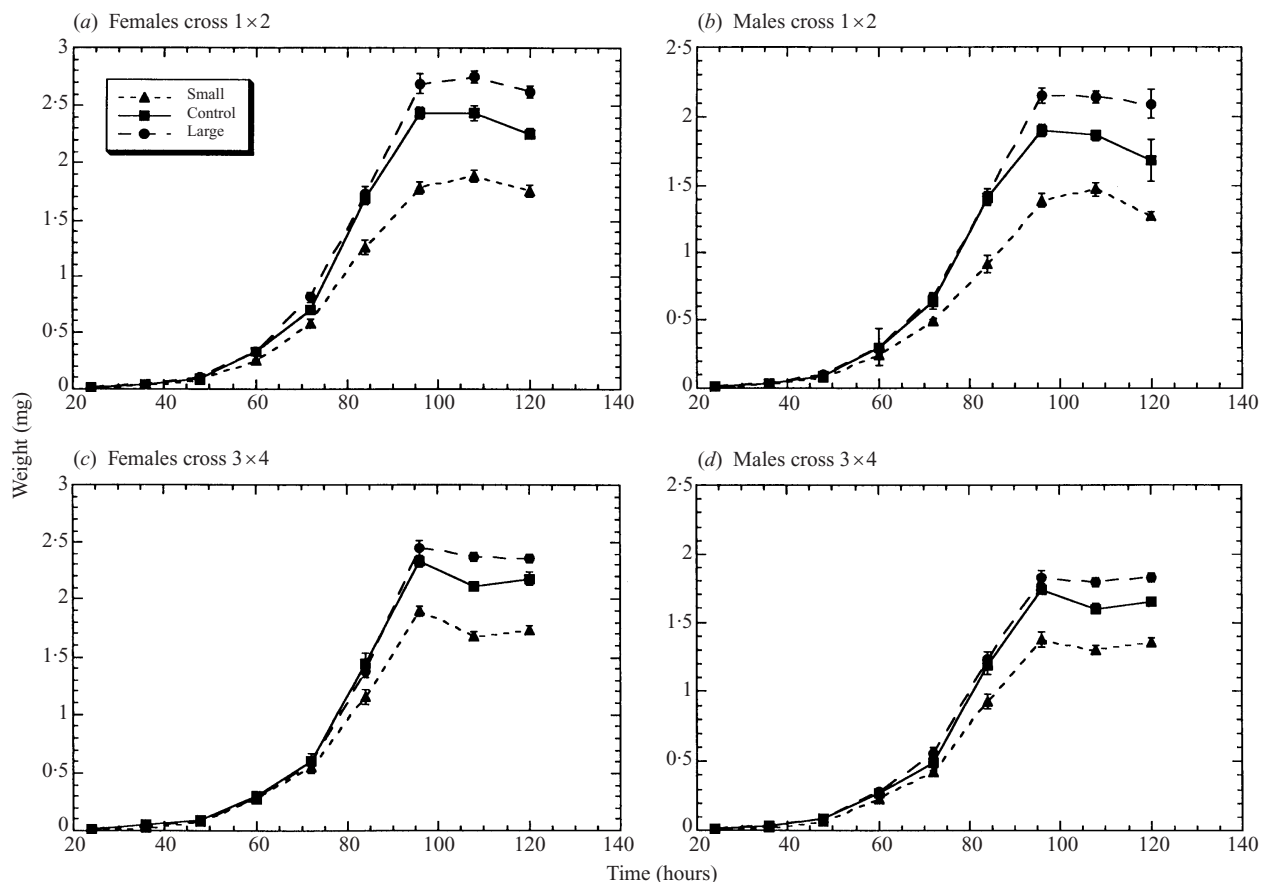


Fig. 2. Larval growth curves (mean and 95% CL weight plotted against age after egg-lay) for the two sets of SMALL, CONTROL and LARGE hybrid 'thorax' selection lines. Data are shown separately for female (a, c) and male (b, d) larvae.

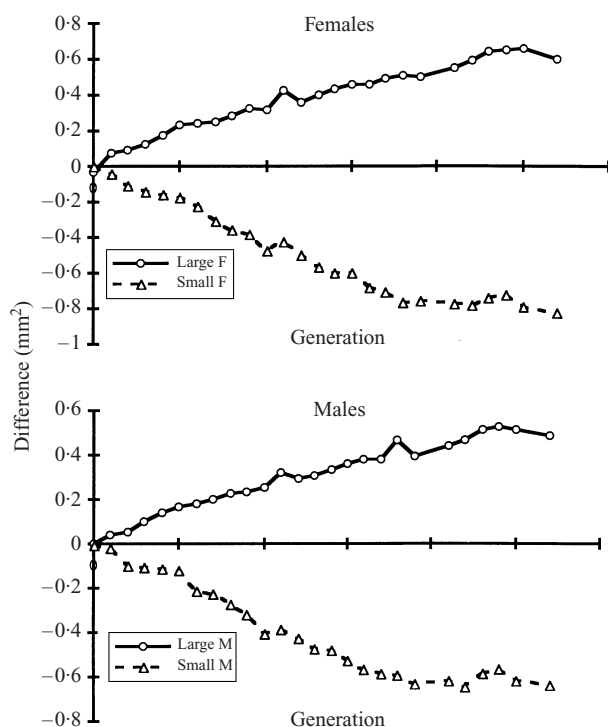


Fig. 3. The response to selection for wing area in the LARGE and SMALL selection lines, plotted as the deviation from controls ( $\text{mm}^2$ ).

sample of the whole growth cohort from the experiment.

The data show that selection in both directions on adult thorax length produced correlated responses in larval growth control and in mature larval body weight.

### (iii) Sizes of fully fed adults

Both sets of 'thorax' hybrid selected lines showed deviations in both female and male thorax length of the LARGE and of the SMALL lines from the CONTROL lines (Table 1). All analyses of variance yielded significant results ( $1 \times 2$  cross, females  $F = 506.9$ , d.f. 2/57,  $P < 0.0001$ , males  $F = 477.9$ ,

d.f. 2/57,  $P < 0.0001$ ;  $3 \times 4$  cross, females  $F = 624.1$ , d.f. 2/57,  $P < 0.0001$ , males  $F = 441.3$ , d.f. 2/57,  $P < 0.0001$ ), and all contrasts were significant at  $P < 0.0001$ . Analysis of variance for wing area also yielded significant results ( $1 \times 2$  cross, females  $F = 264.7$ , d.f. 2/57,  $P < 0.0001$ , males  $F = 467.8$ , d.f. 2/57,  $P < 0.0001$ ;  $3 \times 4$  cross, females  $F = 173.7$ , d.f. 2/57,  $P < 0.0001$ , males  $F = 183.7$ , d.f. 2/57,  $P < 0.0001$ ). Contrasts revealed that the SMALL lines had significantly smaller wings than the CONTROL lines ( $P < 0.001$ ), except for females from the  $1 \times 2$  cross where  $P < 0.01$ . The LARGE line females and males also had larger wings than the CONTROL lines (Table 1), with the differences significant ( $P < 0.01$ ) for the  $1 \times 2$  cross but non-significant for the  $3 \times 4$  cross.

In the 'wing' lines there was a steady response to selection for increased and for decreased wing area (Fig. 3). The mean wing area of the control fluctuated between  $1.52$  and  $1.75 \text{ mm}^2$  for females and between  $1.15$  and  $1.33 \text{ mm}^2$  for males over the course of selection, and the response to selection was very large in relation to this environmental variation in size. When measured at generation 27, large differences in wing area were apparent (for both sexes, all contrasts between selection lines and controls,  $P < 0.001$ ), and correlated responses to selection had also occurred in thorax length and in the wet weights of the LARGE and SMALL line flies (Table 2) (for both sexes, all contrasts between selection lines and controls  $P < 0.001$ ). The body size of the whole fly therefore showed a correlated response to selection on wing area.

### (iv) Cell number and cell size in the wing blade

Estimates of cell size in the wing blade were derived from trichome counts from both sets of 'thorax' selected lines (Table 1). For both cellular traits in both sexes of all hybrid crosses, the results of the analyses of variance were highly significant ( $F$  values, d.f. 2/57, ranged between  $45.6$  and  $281.8$ , all  $P < 0.0001$ ). Inspection of the data showed that the increase in

Table 2. Size traits of lines selected for wing area

Selection:	Small		Control		Large	
	Female	Male	Female	Male	Female	Male
Adult weight (mg)	1.006 (0.0285)	0.620 (0.0146)	1.369 (0.0308)	0.847 (0.0218)	1.509 (0.0395)	1.065 (0.0263)
Thorax length (mm)	0.886 (0.0134)	0.763 (0.0129)	1.015 (0.00966)	0.881 (0.0106)	1.078 (0.00894)	0.950 (0.00708)
Wing area ( $\text{mm}^2$ )	0.827 (0.0132)	0.586 (0.00852)	1.655 (0.0210)	1.290 (0.0177)	2.235 (0.0191)	1.729 (0.0143)
Cell area ( $\text{mm}^2 \times 10^{-4}$ )	0.88 (0.0231)	0.74 (0.0202)	1.64 (0.0346)	1.41 (0.0260)	1.61 (0.0347)	1.43 (0.0261)

Figures are means (and 95% confidence limits).

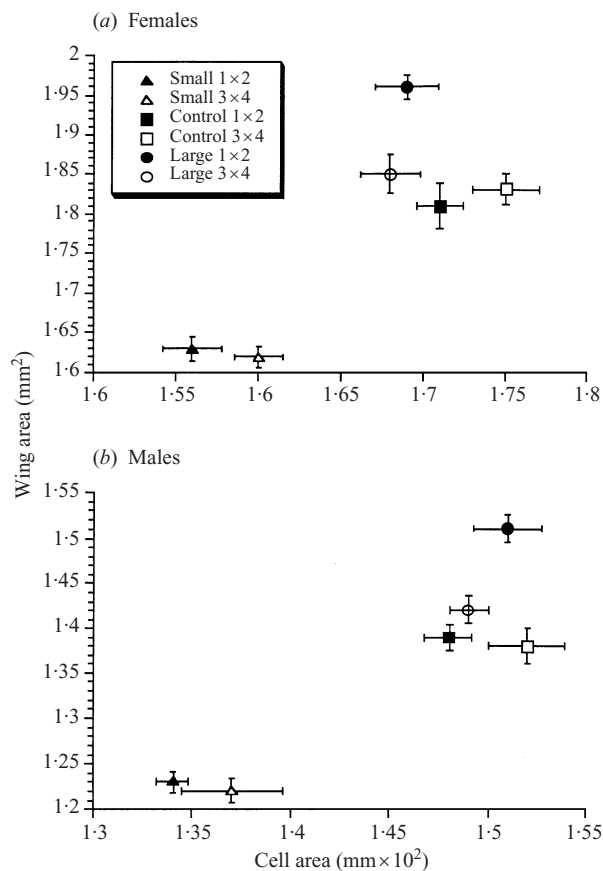


Fig. 4. Wing area (mean and 95% CL) plotted against cell area (mean and 95% CL) for (a) females and (b) males of the two sets of hybrid lines selected for thorax length.

wing area in the LARGE lines was accomplished almost entirely by an increase in the number of cells (Table 1, Fig. 4). The differences in area of both the dorsal and the ventral cells were either non-significant between LARGE and CONTROL lines when contrasts were made (dorsal trichomes for females of the  $1 \times 2$  cross, ventral trichomes for both sexes of the  $1 \times 2$  cross), or were significant but in the wrong direction to explain the difference in wing area, with the LARGE line having smaller cells than the CONTROL flies (dorsal trichomes for both sexes and ventral trichomes for females of the  $3 \times 4$  cross). Only for the males of the  $1 \times 2$  cross was there a significant ( $P < 0.01$ ) difference in dorsal trichome density in the appropriate direction to explain the difference in wing area. Cell area in the wing was greatly reduced in the SMALL lines. For all contrasts, the SMALL lines showed a significant ( $P \leq 0.0001$ ) reduction in wing cell area relative to the corresponding CONTROL lines. To determine whether changes in cell number also contributed, we calculated expected wing areas for CONTROL line flies, assuming that cellular relations in the wing were the same as in the SMALL lines. The predicted values based on dorsal trichomes

were  $1.787 \text{ mm}^2$ ,  $1.359 \text{ mm}^2$ ,  $1.773 \text{ mm}^2$  and  $1.354 \text{ mm}^2$  for females and males of the  $1 \times 2$  and  $3 \times 4$  crosses respectively, and those based on the ventral trichomes were  $1.768 \text{ mm}^2$ ,  $1.360 \text{ mm}^2$ ,  $1.757 \text{ mm}^2$  and  $1.349 \text{ mm}^2$ . In every case these predicted values lie below the lower 95% confidence limit for observed wing area (the exception being the prediction based on dorsal trichomes for females of the  $1 \times 2$  cross). It can therefore be deduced that a reduction in cell number also contributed to the correlated response to selection of wing area in the SMALL selection lines.

In the 'wing' selected lines (Table 2) the LARGE line again achieved its considerable increase in wing size entirely by increasing the number of cells on the wing blade, as the cell areas for both females and males did not differ significantly from the CONTROL lines when tested using linear contrast. As in the 'thorax' lines, the SMALL line showed a substantial and significant ( $P \leq 0.0001$ ) reduction in cell area. Extrapolation from cell numbers in the SMALL line again gave predicted wing areas for CONTROL lines ( $1.541 \text{ mm}^2$  and  $1.117 \text{ mm}^2$  for females and males, respectively) that lay below the lower confidence limit for the observed values, demonstrating that selection for reduced wing area had also resulted in a decline in wing cell number in the SMALL line. These data show that the large direct responses in the 'wing' selection lines were produced in the same way (in terms of effects on cell size and cell number) as the smaller changes in wing size arising as correlated responses in the 'thorax' selection lines.

#### (v) Critical point for pupariation

For the first set of hybrid 'thorax' lines, larvae were starved at 72 h, 76 h and 80 h and, for the second set of lines, at 72 h, 76 h, 80 h and 84 h post-egg lay. Very few larvae (seven in total) starved in the weight categories of 0.6 mg and below produced adults, while over 90% of those starved at 1.2 mg and above in the first experiment and at 1.1 mg and above in the second experiment did so. Also, very few larvae (nine in total) starved at 72 h gave rise to adults. The informative ages were therefore 76 h and 80 h in the first experiment and 76 h, 80 h and 84 h in the second, while the informative weights were 0.7–1.1 mg in the first experiment and 0.7–1.0 mg in the second. The numbers of larvae that were starved and that gave rise to adults in these age/weight categories were subjected to chi-squared analysis (or Fisher exact tests where appropriate) and the results are shown in Table 3. A comparison of the LARGE and CONTROL lines shows that, of 17 age/weight comparisons, six gave significant results, always with the LARGE line larvae less likely to pupariate. A similar comparison for the SMALL lines showed that, of 18 comparisons, five gave significant results, here with the SMALL line



Table 3. Number of larvae (Number of larvae starved giving rise to pupae/total number starved) from lines selected for thorax length and starved at different ages and weights

Age at starvation	Line	Weight at starvation			
		0.7 mg	0.8 mg	0.9 mg	
1 × 2 cross 76 h	S	25/71*	20/41**	10/13 <sup>NS</sup>	
	C	1/12	3/27	28/42	
	L	0/19 <sup>NS</sup>	2/22 <sup>NS</sup>	25/54*	
	80 h	S	8/20 <sup>NS</sup>	26/35	22/24 <sup>NS</sup>
		C	1/1	—	1/2
		L	—	—	0/2 <sup>NS</sup>
3 × 4 cross	76 h	S	3/50 <sup>NS</sup>	10/34 <sup>NS</sup>	11/18 <sup>NS</sup>
		C	2/29	11/46	17/40
		L	0/41 <sup>NS</sup>	4/59*	9/41*
	80 h	S	14/24*	34/42**	21/23*
		C	0/3	5/14	17/26
		L	0/3 <sup>NS</sup>	3/17 <sup>NS</sup>	19/26 <sup>NS</sup>
	84 h	S	—	5/10 <sup>NS</sup>	21/22 <sup>NS</sup>
		C	—	1/5	4/6
		L	—	—	—

S, small; C, control; L, large.

Asterisks indicate significance of the difference of the selection line from the control assessed by chi-squared or Fisher exact tests: \*  $P < 0.05$ , \*\*  $P < 0.01$ .

larvae always significantly more likely to pupariate than the CONTROL lines. Many of the non-significant comparisons in both cases involved either very low numbers of starved larvae from one of the lines, or age/weight categories where very high or very low proportions of the larvae from both lines succeeded in pupariating (Table 3). Given the low sample sizes, the consistency of the direction and the number of significant differences with the use of two-tailed tests, the results of starvation therefore show that the critical age/weight for pupariation had been increased by size selection in the LARGE lines and had been reduced in the SMALL lines.

#### 4. Discussion

Selection for altered thorax length or wing area resulted in a correlated response in total body size, as demonstrated by the changes in mature larval weight (Fig. 3) and adult weight (Table 2). There were also correlated increases in wing area in the ‘thorax’ selected lines (with the exception of the LARGE line in the second cross), and in thorax length in the ‘wing’ selected lines. The change in overall body size in response to selection on the size of a single structure is consistent with the findings of previous artificial selection experiments on *Drosophila* (e.g. Reeve, 1950; Robertson & Reeve, 1952; Cowley & Atchley, 1988, 1990; Wilkinson *et al.*, 1990). We made extensive

measurements, for all the ‘thorax’ hybrid lines, of the thorax lengths of flies developed from larvae starved at known weights (data not shown), and found no evidence of a change in the ratio of thorax length to larval weight. Thus there was no indication that the response to size selection involved any change in efficiency of the conversion, at metamorphosis, of larval weight (comprising energy stores, transient larval tissue and immature imaginal tissue) into mature adult tissue.

The data for larval development time, together with the growth curves (Fig. 3), show that the SMALL ‘thorax’ lines achieved their small size not by curtailing the period of larval growth, but by growing more slowly from a very early age. Even with no other changes, this reduction in growth rate would be expected to lead to a reduced adult body size, provided it persisted into the post-critical period, and it did so. The LARGE lines, however, extended their period of larval growth, rather than increasing their growth rate relative to the CONTROL lines. It is interesting that the LARGE lines did not grow more rapidly, because it is known that larval growth rate can be increased from wild-type values by artificial selection, albeit with an associated decrease in larval survival (Chippindale *et al.*, 1997). An increase in the duration of larval development can also result in decreased larval survival, but only at high levels of larval crowding (Partridge & Fowler, 1993; Santos *et al.*, 1992), which were not used during the production of the selection lines. A decrease in growth rate can also result in reduced larval survival, because the critical weight for pupariation is reached later, possibly resulting in a higher probability of failure to pupariate in more crowded larval cultures (Bakker, 1959). In *D. melanogaster*, increased body size evolves in natural populations at higher latitudes (Stalker & Carson, 1947; David & Bocquet, 1975; Coyne & Beecham, 1987; Imasheva *et al.*, 1993; James *et al.*, 1995; van't Land *et al.*, 1998) and in populations cultured long term at lower temperatures (Anderson, 1966, 1973; Cavicchi *et al.*, 1985; Partridge *et al.*, 1994a), and this increase in size is, in both cases, accompanied by an increase in growth rate and in growth efficiency (James *et al.*, 1995; James & Partridge, 1995; Neat *et al.*, 1995). However, altered intensity of selection on body size *per se* may not be responsible for these changes, and more work is required to understand the natural genetic correlations between size and growth rate. The correlated responses of larval viability to changes in growth rate have not been consistent, which must mean that changes in growth rate can be achieved by different mechanisms. These merit further study. Unlike populations that have evolved larger size at lower temperatures, the lines selected for large thorax length in the present study did not increase larval growth rate, so they must have achieved

increased size through a delay in the triggering of pupariation, an extension of the post-critical feeding period, or both.

Progress through the third larval instar, pupariation and subsequent pupation is controlled by ecdysteroid secretion, which is triggered by release of prothoracicotropic hormone (PTTH) from the brain. In many types of insect, including *Drosophila*, PTTH release occurs after a period of feeding and growth to a certain 'critical weight' (Nijhout, 1994). In many insects, growth and distension of the body may be the direct stimulus (abdominal stretch receptors have been identified in two species of bugs: see Nijhout, 1994), but nutrient levels may also be important (Sehnal, 1985). The direct stimulus for PTTH release has not been identified in *Drosophila*, but the major factor is clearly the state of the larva, not of the imaginal discs. Hence pupariation occurs in larvae that are genetically 'discless' (Szabad & Bryant, 1992) or whose imaginal discs have been eliminated by irradiation (Poody & Woods, 1990). Nonetheless, the rise in ecdysteroid titre and the onset of pupariation can be delayed by continued growth of imaginal discs caused by regeneration after injury or by 'overgrowth' mutations that disrupt growth termination (Sehnal & Bryant, 1993; Simpson *et al.*, 1980). In the present experiments we have studied the triggering of pupariation in relation to larval growth (the 'critical weight') but we could not assess imaginal disc cell number or cell size with sufficient precision to follow the growth of the adult tissue directly (data not shown).

Alterations in critical weight do contribute to the size change in both the LARGE and the SMALL 'thorax' lines. The increase in the LARGE lines was particularly obvious at 76 h when, in most larval weight categories, these larvae were significantly less likely to pupariate than were the CONTROL line larvae. As these lines grow at equal rate, the initiation of pupariation must be delayed in the LARGE lines, resulting in an increase in total larval development time (which was observed) and an increased opportunity for feeding. Interestingly, the SMALL lines had a reduced critical weight and this, in addition to the reduced growth rate, would have contributed to their reduced body size. Because initiation of pupariation depends on both weight and age, and the larvae in the SMALL selection lines grew at a reduced rate, we were unable to assess whether the duration of the post-critical feeding period had changed as a correlated response to selection on adult size. These findings suggest that the critical time or weight for initiating metamorphosis could evolve quite rapidly in other organisms with indirect development (e.g. Bradshaw & Johnson, 1995).

In both the lines selected for thorax length and those selected for wing area, increase in size of the

wing was achieved entirely by an increase in cell number, whereas the SMALL lines showed greatly reduced wing cell size with only a modest effect on cell number. This is a striking and robust result, found both in the replicated 'thorax' selection lines and in the 'wing' lines that were produced at a different time, by selection on a different trait, using a different procedure and starting from a completely different stock population of *D. melanogaster*. The adult wing blade is a very specialized structure, produced by very flattened epidermal cells, and it has been proposed that trichome density gives an estimate of wing cell area that may not reflect cell size in other body regions (Kuo & Larsen, 1987). Recent studies indicate, however, that both environmental and genetic factors produce comparable differences in cell area in the wing and elsewhere (R. Azevedo, V. French & L. Partridge, unpublished data; see also Stevenson *et al.*, 1995). Thus it seems probable that the present results lead to general conclusions on the cellular basis of the response to size selection in *Drosophila*. It is intriguing that a study of Hawaiian giant *Drosophila* species concluded that, in that case, major evolutionary increase in body size has involved a considerable increase in cell size (Stevenson *et al.*, 1995).

During most of larval life the imaginal discs grow exponentially, with a constant rate and fairly uniform distribution of cell divisions and the maintenance of a constant cell size. Prior to pupariation, cell division slows and ceases, as the mature cell number is achieved (see Section 1), and the present results indicate that this 'target' cell number can be decreased or substantially increased by selection for, respectively, small or large body size. The control of cell division is intrinsic to the imaginal disc (Bryant & Simpson, 1984) but it does not involve a determinate mechanism (e.g. each cell undergoing a set number of divisions), as the precise patterns of cell lineage differ between individuals (Bryant, 1996). Furthermore, essentially normal development can occur, despite great alterations in cell proliferation patterns caused by experimental manipulation of the division rate (Morata & Ripoll, 1975; Weigmann *et al.*, 1997) or the survival (Haynie & Bryant, 1977) of some of the cells in the disc.

As the imaginal disc grows, spatial patterns of cell fate become established. Recently it has become clear that genes such as *decapentaplegic* and *wingless* are expressed in thin stripes and their secreted products (Dpp and Wg) form gradients across the disc that control gene expression and future morphological pattern (Nellen *et al.*, 1996; Lecuit *et al.*, 1996; Zecca *et al.*, 1996; Neumann & Cohen, 1997). Growth control is closely integrated with patterning, as ectopic expression of these genes provokes localized cell proliferation and the duplication of pattern (e.g. Zecca *et al.*, 1995) and, conversely, cells that are

unable to transduce these intercellular signals cannot divide (Edgar & Lehner, 1996). It remains unclear just how the largely uniform pattern of proliferation may be controlled by graded signals (discussed by Edgar & Lehner, 1996; Serrano & O'Farrell, 1997). In some situations where cell division is blocked, normal disc size and gene expression patterns may still be achieved, with the formation of giant cells (Weigmann *et al.*, 1997; Johnston, 1998), suggesting that patterning signals may directly affect cell growth. Furthermore, it has been shown that manipulating one component of a common signal transduction pathway can increase or decrease wing size, through effects on both cell size and cell number (Leevers *et al.*, 1996).

The present results demonstrate that selection on body size can alter both cell size and cell number in the wing. It is not clear why the responses to upward and to downward selection should be mediated, respectively, by changes in cell number and in (predominantly) cell size. In nature, the contribution of these cellular traits to size variation in latitudinal clines differs between continents (B. Zwaan, R. B. Azevedo, A. C. James & L. Partridge, unpublished data), suggesting that selection on cell size *per se* may be relatively weak. The cellular basis of the response to selection on body size may therefore depend on the direction of selection and on the relative heritability of cell size and cell number in the direction of selection.

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