

Joint regulation of cell size and cell number in the wing blade of *Drosophila melanogaster*

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Summary

We used *Drosophila melanogaster* to test for compensatory control of cell area and cell number in the regulation of total wing area. In two random bred wild-type base stocks collected from different geographic locations we found a negative association between the area and the number of cells in the wing blade. Three replicate lines were selected for increased or decreased wing area, with cell area maintained at the same level as in the three controls. After eight generations of selection, despite a large and highly significant difference in wing area between the large, control and small selection lines, cell area did not differ significantly between them. Rather, the difference in wing area between selection regimes was attributable to differences in cell number. Over the course of selection, the initially significant negative correlation between cell area and cell number in the wing increased, providing evidence for compensatory regulation of cell area and cell number. As a result of the increasingly negative association between the two traits, the variance in wing area declined as selection proceeded. It will be important to discover the mechanisms underlying the compensatory regulation of cell area and cell number.

1. Introduction

Body size has fundamental consequences for physiology, ecology and life history (e.g. Peters, 1983; Schmidt-Nielsen, 1984; Clutton-Brock, 1988; Stearns, 1992; McNeill Alexander, 1995). There is therefore considerable interest in understanding the mechanisms by which body size is determined. In multicellular organisms, body size has two components: cell size and cell number. In *Drosophila*, both these traits can change during the evolutionary divergence of body size between populations (James *et al.*, 1995) and between different species (Stevenson *et al.*, 1995).

Analysis of the effects of mutants (Spickett, 1963) and of artificial selection (Reeve & Robertson, 1953; Robertson, 1959*a, b*; Masry & Robertson, 1979), and the comparison of *Drosophila* populations (Robertson 1959*a*; Partridge *et al.*, 1994; James *et al.*, 1995) have shown evidence for independent genetic variation for cell size and cell number. Similar findings have been made for the effects of environmental perturbations

on *Drosophila* body size. Low growth temperatures increase body size (e.g. Alpatov, 1930) whilst low levels of nutrition (e.g. Robertson, 1959*a*) or larval crowding (e.g. Atkinson, 1979) decrease it. In the wing, the effect of temperature on size is mediated entirely through changes in cell size (e.g. Alpatov, 1930; Imai, 1934; Robertson, 1959*a*; Delcour & Lints 1966; Masry & Robertson, 1979; Partridge *et al.*, 1994) while, at high levels of crowding and under poor nutritional conditions, size is decreased through reduction in both cell size and cell number (e.g. Robertson, 1959*a*).

The adult head and thorax of *Drosophila* are formed from discrete imaginal discs that grow through larval life and replace the larval epidermis at metamorphosis. There is evidence that the cell number in imaginal discs is strongly regulated. If pupariation is delayed by early damage to some of the discs, no part of the resulting fly becomes abnormally large, indicating that undamaged discs do not grow beyond their normal size, despite the extension of larval life (Simpson *et al.*, 1980). Similarly, immature imaginal discs removed and cultured *in vivo* in an adult abdomen will continue to grow, but will not exceed approximately the normal disc cell number (Bryant &

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Levinson, 1985). Hence control of cell number may be largely intrinsic to the imaginal discs, given adequate larval nutrition (Bryant & Simpson, 1984). The disc-intrinsic control of cell proliferation is likely to operate via local cell interactions, rather than by assessment of a 'target' total cell number for the disc (see Bryant & Simpson, 1984; Bryant, 1987; French, 1989). It seems that disc cell division continues and normal cell numbers are exceeded only when spatial patterning is disrupted. This can occur either in overgrowth mutants (Bryant & Levinson, 1985) where pupariation is delayed or prevented (Sehnal & Bryant, 1993), or when extra structures are formed following, for example, the experimental mis-expression of patterning genes such as *decapentaplegic* (e.g. Nellen *et al.*, 1996). The regulation of cell size is not well understood. *Giant* mutations increase body size and, in the wing, they act by increasing cell area (Simpson & Morata, 1980), probably by inducing errors in DNA replication and hence abnormal ploidy, which has previously been documented as a cause of abnormal cell area (Dobzhansky, 1929; Lindsley & Zimm, 1992).

It is important to discover whether the regulation of total body size involves compensatory regulation of cell size and cell number, or whether these are independently controlled. Recent transgenic manipulations in *Arabidopsis* have indicated that reduced cell number may be associated with increased cell area (Hemerly *et al.*, 1995) but, in another study, increased cell number and no change in cell area resulted in a slightly larger plant (Doerner *et al.*, 1996). It has long been known that salamanders with increased ploidy have larger but fewer cells, resulting in approximately normal total body size and organ size (Fankhauser, 1945, 1955). *Drosophila* triploids have increased cell size but the body size is not increased in proportion with cell size (Lindsley & Zimm, 1992) and, in the leg, there is evidence of compensation through a reduction in cell number (Held, 1979). Artificial selection on cell area in the wing of *Drosophila* produced conflicting results, with both positively and negatively correlated responses in cell number being observed (Robertson, 1959*a*). Unfortunately, these selection lines were unreplicated.

In this paper we describe an experiment with *D. melanogaster* in which we analysed compensatory control of cell area and cell number in the regulation of total area of the wing blade. We first established that in two different base stocks there was a negative correlation between the two traits across individuals, suggesting compensation. Using one of these base stocks, we then selected for increased and decreased cell number in the wing blade, while holding cell area constant, and examined the consequences for the correlation between cell area and cell number across individuals. We reasoned that, if wing area *per se* is regulated, the level of compensation between cell area and cell number might increase as selection proceeds.

2. Materials and methods

Two outbred wild-type base stocks collected in different geographic areas were used to examine the relationship between cell area and cell number. The first (Brighton) was collected in Brighton fruit market, England, in 1984, while the second (Dahomey) was collected in Dahomey, West Africa, in 1970. Both have since been maintained in mass culture in population cages at 25 °C.

(i) Wing area and cell area measurements

Measurements were made on flies derived from the Brighton stock and reared under standard conditions. Eggs were collected from the population cage in yeasted bottles of medium, reared at 25 °C and the resulting adults transferred to 'laying pots' containing a yeasted grape juice medium. After 24 h for the adults to acclimate and a 3 h pre-lay period (for oviposition of any retained eggs), the flies were given fresh medium, and eggs collected after 3 h. Upon hatching, 150 first instar larvae were placed singly in vials containing 7 ml medium seeded with live yeast, and reared at 25 °C. The emerging flies were collected and frozen. The Dahomey 'base stock' measurements were derived from all individuals in the initial generation of the selection lines, before selection was applied (see below).

One wing was removed from each fly, fixed in propanol and mounted in Aquamount on a microscope slide. Wing area was measured using a camera lucida attached to a dissecting microscope and a Quora graphics tablet connected to an Apple Macintosh II computer. The outline of each wing was traced starting at the alar–costal break (Fig. 1). Using a compound microscope with a camera lucida attachment, the trichomes were counted within a 0.01 mm² area of the posterior medial cell of the wing (Fig. 1). Average cell area was estimated by dividing 0.01 mm² by the trichome count.

(ii) Selection for wing area

The Dahomey stock was used to produce three replicates of large, control and small lines which were artificially selected for wing area, while maintaining a constant cell area. To produce the parents for the selected lines, eggs were collected from the population cage, hatchling larvae were reared at a density of 50 larvae per yeasted vial, and the emerging adults were collected as virgins. The nine lines were each founded with 10 randomly chosen pairs which were transferred to laying pots, and eggs were collected after 6 h. For each replicate line, 100 first instar larvae were placed in a bottle of 70 ml medium seeded with live yeast.

The first 25 pairs of adults to emerge from each bottle were collected as virgins and stored at 18 °C, two same-sex flies per vial. Under CO₂ anaesthesia,

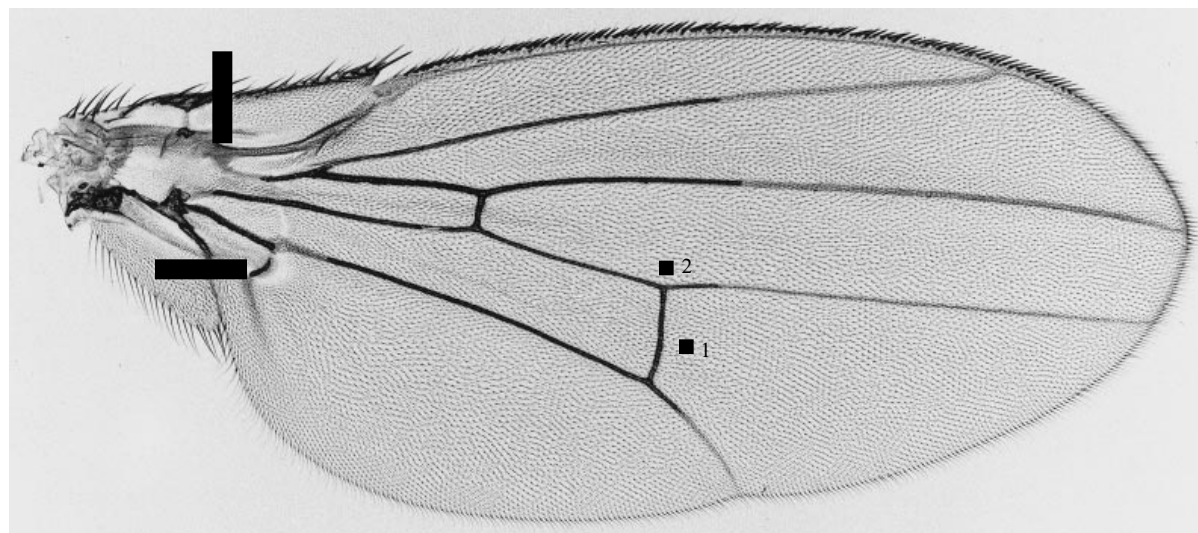


Fig. 1. Diagram of the wing of *D. melanogaster* indicating the region used for wing area measurement (limits shown by bars), and region 1 and region 2 where trichome counts were made for estimating cell area (shown by filled squares). On wings of different sizes, the regions were chosen by eye to correspond to the same locations with respect to veins and the wing margin. During the course of selection, trichome counts were made in region 1. In generation 8 trichome counts were made in both regions.

one wing was removed from each fly, fixed, mounted and scored for wing area and cell area. All the measurements of cell area and wing area from the flies from the nine bottles in the parental generation were pooled to provide the Dahomey base stock measurements.

From each line, 10 pairs of flies were selected as the parents of the next generation. For each sex of each of the three replicate large lines, the data were sorted according to wing area, from largest to smallest, and the mean cell area of the first 10 flies was calculated. If this was greater than the overall mean cell area of the control lines for each sex, then the largest fly was excluded and the fly with the eleventh largest wing area was included in its place. This process was continued until a threshold was reached where the inclusion of the fly with the next smallest wing would have taken the mean cell area of the 10 included flies just below the mean cell area of the controls. In alternate generations, the selected flies of each sex did include the fly that took them just below the control mean cell area. For the three replicate small lines the process was the same, except the data for each sex were sorted from smallest to largest wing area. For the three control lines, 10 males and 10 females were selected at random. The selected flies were transferred to laying pots and returned to 25 °C for mating and collection of eggs. The next generation of each replicate line was produced from 100 first instar larvae reared in a bottle.

After eight generations of selection, cell area was measured in two standard wing regions (see Fig. 1). Two regions were sampled because cell density varies over the wing, and the relative sizes of different parts of the wing can be altered by artificial selection (Weber, 1992). It was therefore important to establish

whether selection had affected cell area in the same way in different wing regions.

Eggs were collected from each replicate line and cultured under relaxed larval competition in bottles. The resulting adults were allowed to lay eggs, and first instar larvae were transferred, at a density of 30, to 20 yeasted vials per replicate line. The adults were collected and frozen. Five individuals of each sex were chosen randomly from each replicate vial and one wing from each fly was removed, mounted and measured for wing area and cell area in each of the two wing regions. In addition, the thorax length of each fly was measured in order to investigate whether selection had resulted in a general change in body size. Individuals were placed on their right sides under a dissecting microscope with an eyepiece graticule, and the length of the thorax was estimated to the nearest 0.02 mm from the base of the most anterior humeral bristle to the posterior tip of the scutellum.

3. Results

(i) Brighton and Dahomey base stocks

Following the approach of Robertson (1959a) and Stevenson *et al.* (1995), the relative contribution of cell area to phenotypic variation in wing area was investigated by linear regression of log-transformed data. If cell area and cell number tended to vary in parallel, then the slope of log wing area on log cell area would be greater than 1, while if they varied independently the slope would be not significantly different from 1. A negative relationship between cell area and cell number would produce a slope of less than 1, approaching 0 with greater levels of compensation. The slopes of the regressions of log wing area on log cell area were significantly less than 1 for

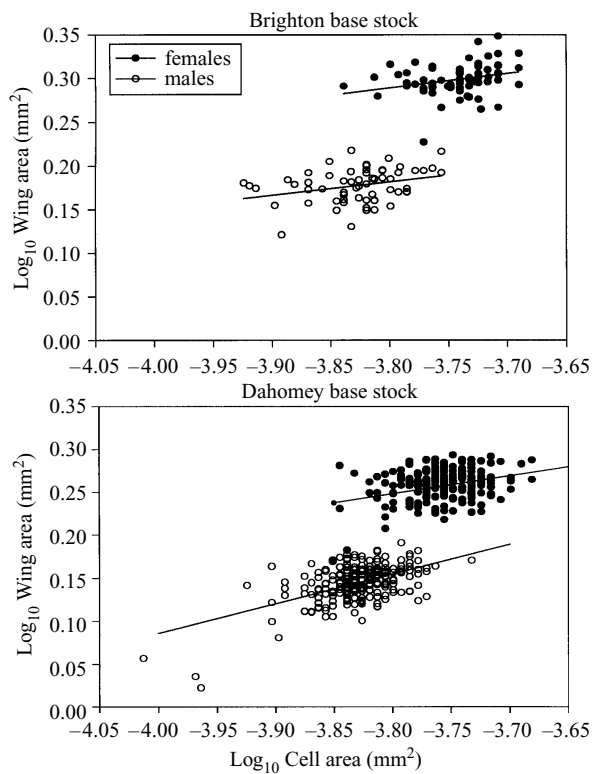


Fig. 2. The relationship between \log_{10} cell area and \log_{10} wing area for males and females from the Brighton and Dahomey base stocks. For both sexes from the two stocks the slope was significantly less than 1, giving evidence for compensation (Brighton males: slope = 0.153 ± 0.0627 , $t = -13.5$, $n = 62$, $P < 0.0001$; Brighton females: slope = 0.165 ± 0.0694 , $t = -12.03$, $n = 63$; $P < 0.0001$; Dahomey males: slope = 0.346 ± 0.0377 , $t = -17.35$, $n = 225$, $P < 0.0001$; Dahomey females: slope = 0.265 ± 0.039 , $t = -18.84$, $n = 225$, $P < 0.0001$). The measurements of the Brighton stock were taken from 62 males and 63 females reared at a density of one larva per vial. The 225 males and 225 females from the nine bottles in the parental generation of the selection experiment were pooled to provide the Dahomey base stock measurements.

males and females from both the Brighton and the Dahomey base stocks (Fig. 2), giving evidence for compensation as a consequence of regulation of total wing area.

(ii) Selection lines

The wing areas of the first 25 males and females emerging in the large and small selected lines showed a steady divergence from the control lines in each generation (Fig. 3). Analysis of variance, with selection regime as a fixed effect and replicate lines nested within selection regimes, showed that the difference between the large and control lines first became statistically significant ($P < 0.05$) in females in generation 3 and in males in generation 4. The corresponding time for both sexes in the small lines was generation 4 ($P < 0.001$). The wing area of the control lines fluctuated between generations, but linear re-

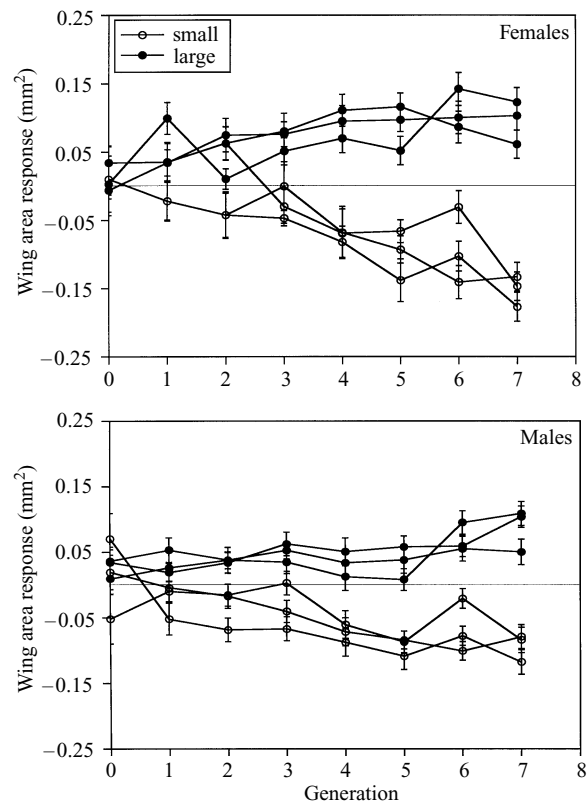


Fig. 3. The response to selection for increased and decreased wing area in lines produced from the Dahomey base stock. The generation means for each of the three replicate large and small selection lines are shown as deviations from the overall mean of the three control lines. Each value is based on the measurements of 25 individuals of each sex from each line. Error bars represent the 95% confidence limits of the mean for each replicate large and small selection line in each generation.

Table 1. Realized heritabilities for upward and downward selection on wing area with constant cell area, calculated as the mean of the regressions of the wing area response on cumulated selection differential for each replicate selection line

	$h^2 \pm SE$		
	Large	Small	n
F + M	$0.42 \pm 0.07^{***}$	$0.47 \pm 0.06^{***}$	3
F	$0.43 \pm 0.10^*$	$0.46 \pm 0.04^{***}$	3
M	$0.41 \pm 0.14^*$	$0.48 \pm 0.13^*$	3

* $P < 0.05$; *** $P < 0.001$.

Standard errors were calculated from the observed variance of the regression coefficients between replicates (Hill, 1972). F, females, M, males, F + M, offspring mean. A one-tailed t-test was performed to test whether the heritability value was significantly different from zero.

gression showed no significant trend over generations. Despite the constraint on cell area, selection yielded large and significant realized heritabilities for wing area (Table 1).

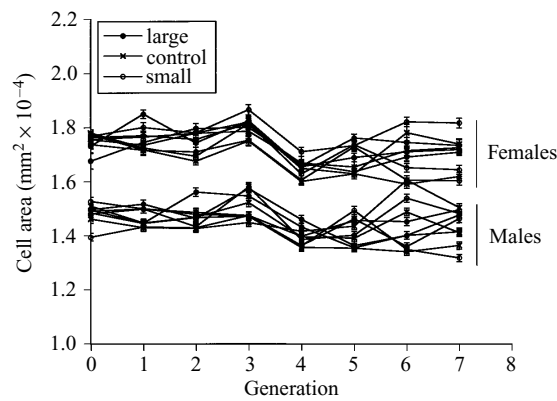


Fig. 4. Cell area over the course of selection. Mean cell area (and 95% confidence limits) of the wings of males and females from the three replicate large, control and small wing area lines are plotted against generation. In each generation 25 males and 25 females were measured from each selection line.

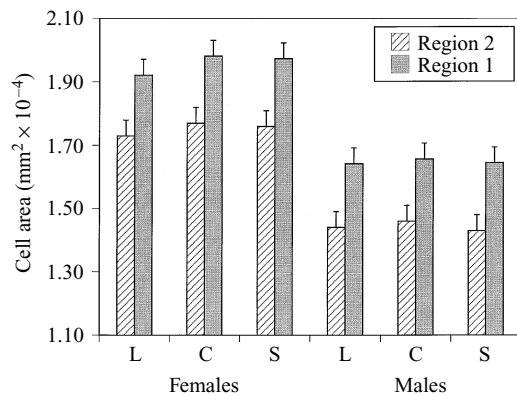


Fig. 5. Mean and 95% confidence limits for the cell area in wings of flies from the three replicate large (L), control (C) and small (S) selection lines in generation 8. Cell area was measured in two regions of the wings of 5 males and 5 females from each of 20 vials for each selection line. The mean and 95% confidence limits were calculated from the vial means. There was no significant difference in cell area between the selection regimes (region 1: $F_{(2,6)} = 3.28$, NS; region 2: $F_{(2,6)} = 3.18$, NS), nor any significant interaction between sex and selection regime (region 1: $F_{(2,6)} = 1.09$, NS; region 2: $F_{(2,6)} = 1.10$, NS).

There were no significant differences in cell area between flies from the different selection regimes in any generation nor any significant trends in cell area over generations (Fig. 4). This lack of a difference in cell area between selection regimes was confirmed when both regions of the wing were measured in generation 8 (Fig. 5). Females had significantly larger cells than males, but there was no indication of an interaction between sex and selection regime. There was a highly significant ($P < 0.0001$) effect of selection regime on both wing area and thorax length as measured in generation 8 (Fig. 6). It therefore follows that selection caused an overall change in body size and that the changes in wing area produced by selection were entirely due to changes in cell number.

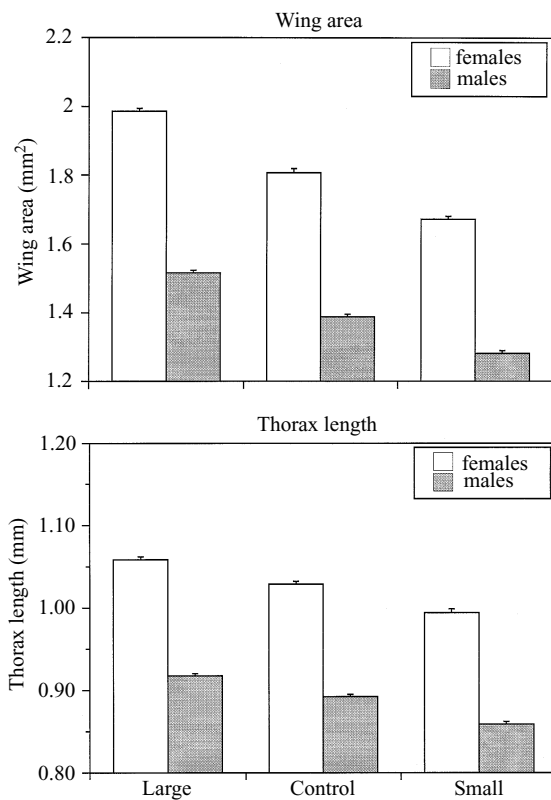


Fig. 6. Mean and 95% confidence limits for the mean wing area and the mean thorax length of flies from the large, control and small selection lines in generation 8. Five males and 5 females were measured from each of 20 vials for each replicate selection line, and the mean and 95% confidence limits were calculated from the vial means. There was a highly significant effect of selection regime on both wing area ($F_{(2,6)} = 46.1$, $P < 0.0001$) and thorax length ($F_{(2,6)} = 38.8$, $P < 0.0001$).

For the control lines, the slopes of the regressions of log wing area on log cell area fluctuated between 0.22 (SE 0.07) and 0.34 (SE 0.064) over the course of selection (Fig. 7), with all values significantly less than 1 ($P < 0.0001$). The slopes of the regressions for the large and small selection lines, however, declined over the course of selection (Fig. 7). In generation 8, the slope was significantly less than 1 for the control lines and not significantly different from 0 for either the large or the small selection lines (Fig. 8). Thus there was evidence for increasing levels of compensation between cell area and cell number as selection on total wing area proceeded.

Regression of the variance of wing area on generation of selection revealed that, for both large and small selection lines, variance in wing area declined over the course of selection (large females: $F_{(1,6)} = 7.497$, $P < 0.05$; large males: $F_{(1,6)} = 7.000$, $P < 0.05$; small females: $F_{(1,6)} = 7.210$, $P < 0.05$; small males: $F_{(1,6)} = 6.3065$, $P < 0.05$). In contrast, there was no trend in the variance in cell area for either the large or small selection lines (large females: $F_{(1,6)} = 1.738$, NS; large males: $F_{(1,6)} = 3.5681$, NS; small females: $F_{(1,6)} = 0.252$, NS; small males: $F_{(1,6)} = 0.513$, NS). The

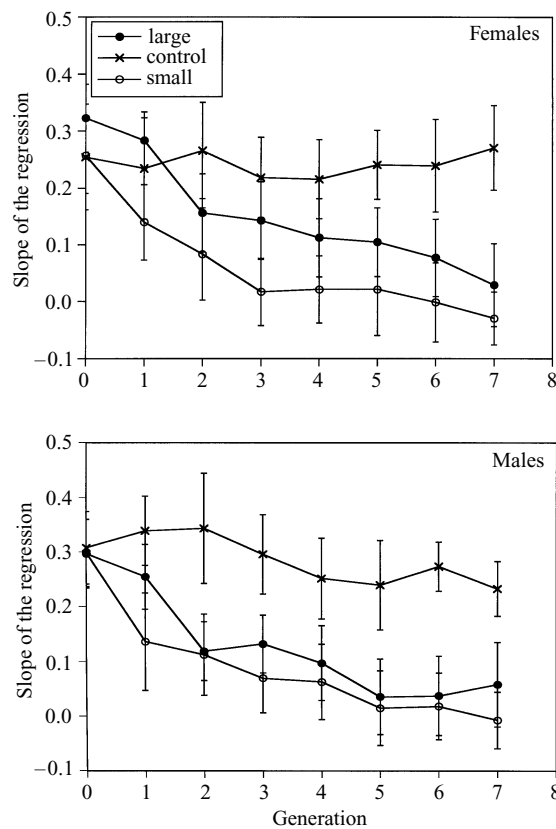


Fig. 7. The slope (and standard error) of the linear regression of \log_{10} cell area and \log_{10} wing area for males and females of the large, small and control selection lines in each generation of selection. Each point is derived from the regression for the 75 individuals representing the pooled replicate lines of each selection regime. The slopes for the large and small lines were no longer significantly different from zero by generation 5 and 4 respectively (large females: $t = 1.72$, d.f. = 73, NS; large males: $t = 1.4$, d.f. = 73, NS; small females: $t = 0.29$, d.f. = 73, NS; small males: $t = 1.1$, d.f. = 73, NS).

available variation in total wing area for selection to act upon therefore declined as selection proceeded, although the variance in cell area did not.

4. Discussion

Our most important finding was a negative association between cell number and cell area in the wing blade. This result provides evidence that the overall area of the wing blade (or of its components) is controlled, irrespective of the pattern of cellularization. Given optimal nutrition and sufficient time to complete development, the cell number in adult imaginal disc derivatives is controlled by local cell-to-cell interactions (Bryant, 1987; Bryant & Simpson, 1984; Bryant & Schmidt, 1990). In the wing disc, *decapentaplegic* is expressed in a thin medial stripe, and the secreted gene product (a TGF- β growth factor homologue) controls proliferation and cell patterning (Zecca *et al.*, 1995). The effects appear to extend over a distance corresponding to 5–10 cell diameters from

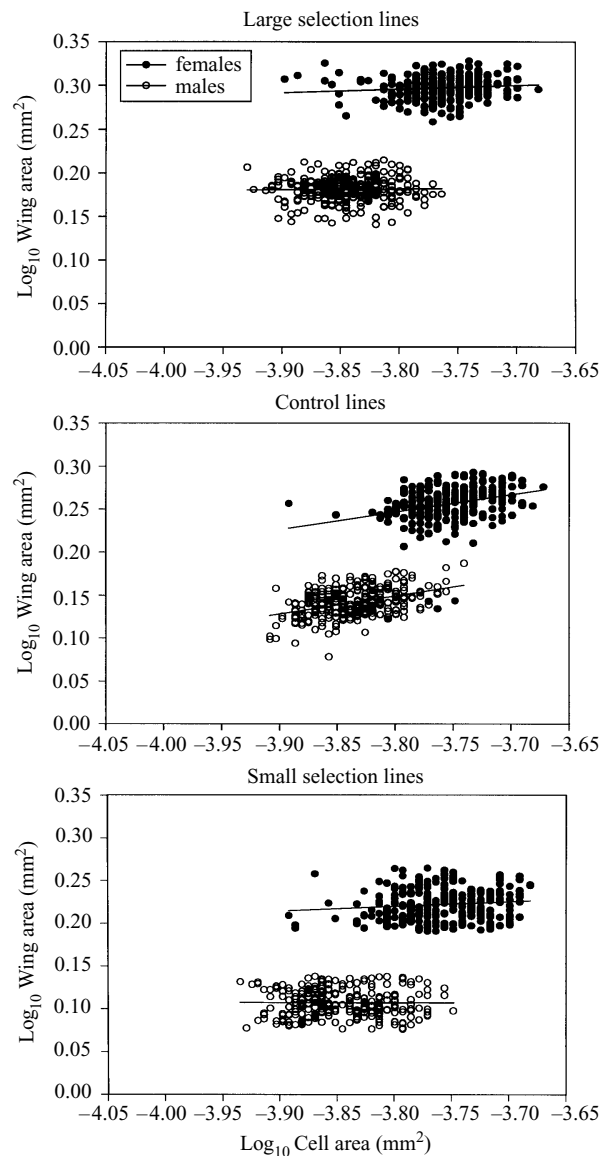


Fig. 8. The relationship between \log_{10} cell area and \log_{10} wing area for males and females of the large, control and small selection lines in generation 8. The selection lines were produced from the Dahomey base stock. Three hundred males and 300 females were measured from each selection regime.

the source of the signal (Nellen *et al.*, 1996; Lecuit *et al.*, 1996; Burke & Basler, 1996). Control of the overall area of the tissue implies that DPP (and other extracellular signals acting on cell division) span a certain absolute distance, rather than, in some sense, ‘counting cells’.

In addition, we found increased levels of compensation between the number and size of cells as wing area deviated more strongly from that of the base stock. The response to selection for wing area through altered cell number therefore occurred in the face of an opposing mechanism for control of total wing area, and resulted in declining variance in total wing area. Selection evidently changed the disc cell interactions that control proliferation and it would be extremely interesting to know how this enhanced the

compensation between cell size and number. One way to explore this would be to select on cellular relations in sub-areas of the wing, and to observe the correlated response over the whole wing blade. To find the genes involved, it would be necessary to find mutants that cause a breakdown in the correlations that we have observed across individuals.

There was significant heritability for wing area, with an average realized heritability of 0.45. This is likely to be an underestimate of the total genetical variation for wing area because of the constraint on cell area. Indeed, artificial selection on wing area alone yielded larger realized heritabilities for wing area than those reported here, with an average value of 0.6 over eight generations of selection (B. Zwaan & L. Partridge, unpublished data).

Artificial selection on thorax length (L. Partridge, K. Fowler, R. Langelan & V. French, unpublished data) and wing area (B. Zwaan & L. Partridge, unpublished data) can cause an asymmetrical response in the cellular mechanism for changing body size, with larger size being due to more cells and smaller size due to decreased cell area, at least as assessed in the wing blade. Our present results could help to explain this asymmetry. If increased cell number is more heritable than increased cell area then, as cell number increased, compensation would tend to hold cell area down. If decreased cell area is more heritable than decreased cell number, a similar effect would be observed. We know of no data, however, on the relative heritabilities of the two traits in the upwards and downwards directions.

Understanding the mechanisms of control of body size, cell size and cell number, in *Drosophila* and elsewhere, provides an important challenge for future work.

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