



Competition among growing organs and developmental control of morphological asymmetry

Christian Peter Klingenberg and H. Frederik Nijhout

Department of Zoology, Duke University, Durham, NC 27708-0325, USA (cpk@acpub.duke.edu)

Fluctuating asymmetry is often used as a measure of developmental instability, although its developmental basis is poorly understood. Theoretical models and experimental studies have suggested that feedback interactions between structures on the left and right sides of the body play a pivotal role in the control of asymmetry. Here we provide experimental evidence that competition for a limiting resource can generate such interactions between growing organs. In our experiments in the butterfly *Precis coenia* (Lepidoptera: Nymphalidae), hindwing imaginal discs were removed from one or both body sides of caterpillars. Emerging butterflies were thus missing one or both hindwings, but had heavier forewings, mid- and hindlegs than untreated controls. When only one hindwing was removed, the forewing and hindleg on the treated side were heavier than on the untreated side. The asymmetry and overall weight increase in response to wing disc removal diminished with increasing physical distance of the responding tissue from the imaginal disc removed. Our findings are consistent with the hypothesis that growing imaginal discs compete for a haemolymph-borne resource, such as a nutrient or growth factor. Such competition is a possible mechanism for feedback interactions and may thus participate in the developmental control of asymmetry.

Keywords: competition; development; fluctuating asymmetry; growth; *Precis coenia*; wing disc

1. INTRODUCTION

Fluctuating asymmetry, small random differences between body sides in otherwise bilaterally symmetrical organisms, has been widely used as a measure of developmental instability (Palmer & Strobeck 1986; Markow 1995; Møller & Swaddle 1997). Its developmental origin, however, is mostly unclear. There is only one case in which specific genes with a major influence on non-directional asymmetry have been identified (Davies *et al.* 1996; Batterham *et al.* 1996), and another study reported a number of quantitative trait loci (Leamy *et al.* 1997). But even in these examples, the role of these genes in the development of asymmetry is not fully understood.

A common feature of the various hypotheses of developmental mechanisms that establish symmetry or asymmetry is feedback between developing organs on opposite body sides, for instance a 'localised, or left-right, signalling system which monitors and regulates morphogenesis' (Swaddle 1997, p. 59). The importance of these feedback mechanisms has been demonstrated by theoretical modelling (Graham *et al.* 1993), but the empirical data are inconclusive. One observational study, measuring fluctuating asymmetry in successive growth stages of the same individuals, found a decrease in asymmetry over time, and thus suggested possible feedback regulation (Swaddle & Witter 1997), whereas two other studies did not find similar patterns (Chippindale & Palmer 1993; Møller 1996). Experimental evidence for feedback mechanisms comes from studies in lobsters and

crabs, where differential use of claws, under the control of the central nervous system, produces pronounced asymmetry (Govind & Pearce 1992; Smith & Palmer 1994). Asymmetries due to differential use of structures also have been shown in other organisms, including humans (Trinka *et al.* 1994). In many other cases, however, asymmetries develop before organ primordia are in use. Hence, left-right feedback, if it exists, must rely on a different, unknown mechanism. Finally, the idea of left-right feedback is somewhat at odds with current paradigms in developmental biology, because the fine-tuning of growth relevant for these asymmetries is thought to be controlled within the growing organs themselves, and largely independent of systemic regulation (Bryant & Simpson 1984; Bryant & Schmidt 1990).

Here we present experimental evidence suggesting that such a systemic feedback interaction can be produced by competition among growing body parts for a resource limiting growth. Competition between growing organs has been explored in theoretical models (Nijhout & Wheeler 1996) and has been used to explain observed correlations among adult structures (Kawano 1997). Recently, Nijhout & Emlen (1998) experimentally showed trade-offs in resource allocation to morphological structures in beetles and butterflies. In their experiment with the buckeye butterfly (*Precis coenia*), they showed that removal of hindwing imaginal discs from last instar caterpillars resulted in an increase in the weight of the forewings and other structures. Here we extend these results and show that competition for the limiting resource

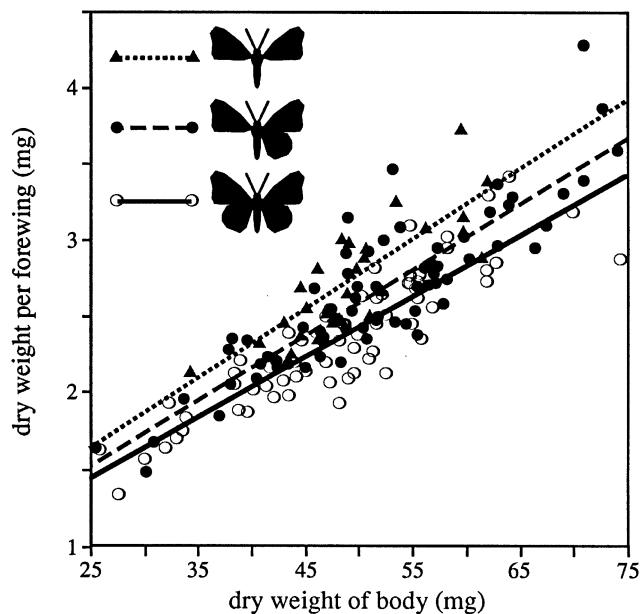


Figure 1. Regression of dry weight per forewing (the average of the left and right sides) on dry weight of the body for butterflies from which both, one, or no hindwing imaginal discs had been removed.

is sufficiently localized that removal of only one hindwing imaginal disc results in an increased mass of structures on the treated relative to the untreated body side. Therefore, competition between growing organs for a limiting resource is a possible feedback mechanism contributing to the control of morphological asymmetry.

2. MATERIAL AND METHODS

For our experiments, we used a laboratory colony of the buckeye butterfly (*Precis coenia*; Nymphalidae), derived from butterflies caught in the wild near Durham (North Carolina, USA). Caterpillars were fed *ad libitum* with an artificial diet supplemented with the host plant (*Plantago lanceolata*, Plantaginaceae). On the day of the moult from the fourth to the fifth (final) larval instar, we allocated caterpillars to one of five treatments, and performed surgery on imaginal discs.

The experimental treatments were the following: (1) removal of the left hindwing imaginal disc; and (2) removal of the right

hindwing disc. The hypothesis of competition predicts that the forewing and legs on the treated side should be larger than on the untreated side, where the remaining hindwing competes for resources. As controls, we used (3) removal of both hindwing discs to examine the effect of disc removal *per se* on asymmetry of other structures, and sham treatments with (4) injury of the left side of the prothorax and (5) injury of the right side of the prothorax to assess the effects of one-sided injury (not affecting the wing discs) on the growth of nearby structures. The hypothesis of competition predicts no directional asymmetry of forewings or legs in the control treatments (3)–(5).

After surgery, caterpillars were reared to adulthood. Mortality immediately following surgery was low, and most deaths occurred during the pupal stage; total mortality ranged from 5% (treatment 1) to 25% (treatment 4). This indicates that the direct effect of surgery (e.g. haemolymph loss) or disc removal *per se* were not major causes of mortality. The number of emerging butterflies was 40 for treatment (1), 32 for (2), 27 for (3), 33 for (4) and 40 for treatment (5). Butterflies were killed by deep-freezing several hours after emergence, when the cuticle had hardened.

The forewings were cut carefully from the butterflies with scissors under a dissecting microscope, and the middle and hind legs were removed with forceps. Wings, legs, and the body were stored separately, dried, and weighed with a Cahn electrobalance (to the nearest 1 µg for wings, 0.1 µg for legs, and 10 µg for bodies). Wings or legs contaminated with body fluids were excluded, because this can artificially increase the weight. The wings and legs of the 15 butterflies weighed first were reweighed at the end of the series. This showed that weighing error and changes in the water content were negligible relative to true asymmetry and error presumably introduced by removing wings and legs from the butterflies. Although this was done by the same person and with the greatest care, it is possible that there was subtle asymmetry because of the way the butterfly and scissors were held (e.g. handedness of the person and of the scissors) or because of genuine directional asymmetry. Because this problem should affect all treatments equally, the hypothesis of competition can be tested by a comparison between means of contralateral treatments (1 versus 2 and 4 versus 5) rather than by a test of the deviation from symmetry for each treatment. Forewing area was measured with a video system; repeatability of area measurements was extremely high, and as for weight, variation in the way the wings were cut off was presumably the dominant source of error.

There was no sex dimorphism in the data considered here after correction for size effects; hence, no extra adjustments for

Table 1. *Effects of hindwing disc removal on the size of other body parts (average of left and right body sides)*

(The upper part of the table gives the overall *F*-statistic and significance level for the effect of treatments from an analysis of covariance (with dry body weight as the covariate). The lower part presents treatment effects as least-squares means (\pm standard error), i.e. estimated treatment means corrected for the effect of body size, and significance levels from pairwise comparisons (one-tailed *t*-tests). Significance levels are not adjusted for multiple comparisons.)

	forewing weight (mg)	forewing area (mg ²)	midleg weight (mg)	hindleg weight (mg)
<i>F</i> (d.f.)	31.8 (2, 180)	0.58 (2, 154)	7.17 (2, 176)	20.1 (2, 175)
<i>p</i>	0.0001	0.56	0.001	0.0001
no disc removed	2.41 \pm 0.02	234.1 \pm 2.0	0.400 \pm 0.004	0.365 \pm 0.003
<i>p</i>	< 0.0001	—	0.026	0.029
one disc removed	2.59 \pm 0.03	236.2 \pm 2.3	0.411 \pm 0.004	0.375 \pm 0.004
<i>p</i>	0.0002	—	0.012	< 0.0001
two discs removed	2.77 \pm 0.04	239.0 \pm 4.4	0.428 \pm 0.007	0.406 \pm 0.006

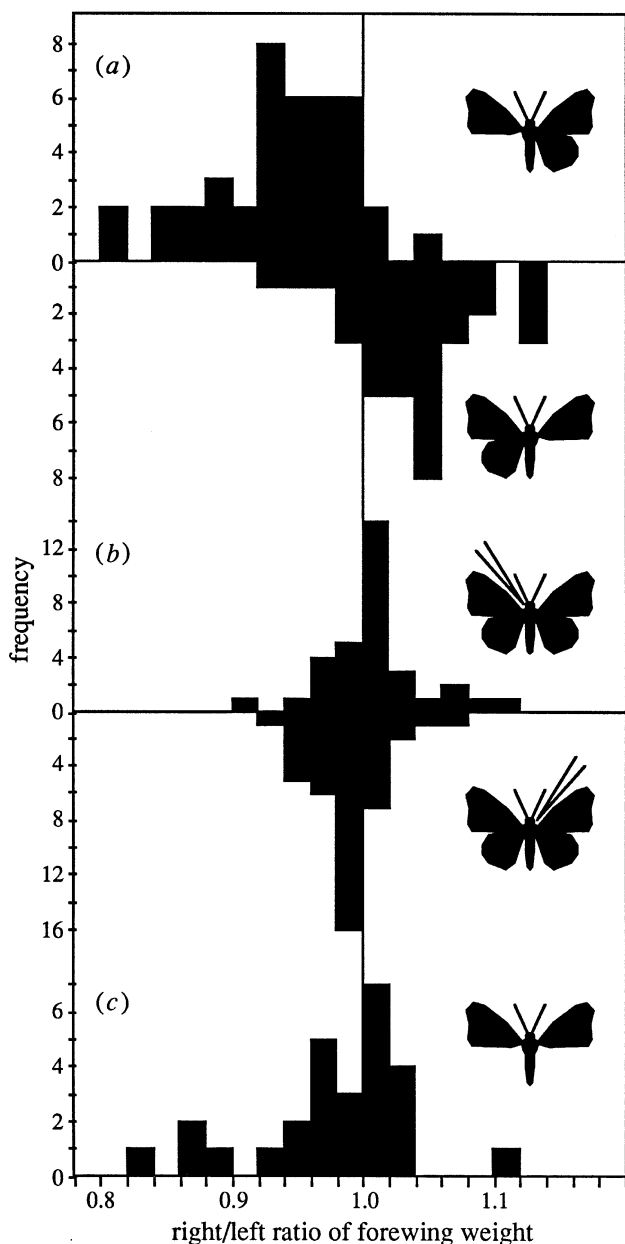


Figure 2. Frequency histogram of the right/left ratio for forewing weight in the different treatments. (a) Experimental treatments, in which the left or right hindwing disc was removed. (b) Sham treatment, in which the prothorax was injured. (c) Control for the effect of disc removal, in which both hindwing discs were removed.

sex were necessary. The right/left ratios were not significantly correlated with either the right/left mean of the respective variable or the dry weight of the body. Therefore, we use uncorrected right/left ratios in our analyses.

3. RESULTS

The size of wings and legs was dependent on dry body weight, as is evident from regression plots (figure 1). Analysis of covariance, with dry weight of the body as the covariate, indicated that there were no significant differences in slope for any of the measurements, but significant differences in means among treatments for three of the four variables (table 1). For a given size,

forewings and legs tended to be largest after removal of both hindwing discs, intermediate for specimens with one hindwing left, and smallest for controls with both hindwings (figure 1, table 1). This effect was strong for forewing weight and hindleg weight, less so for midleg weight, and weak and statistically non-significant for forewing area.

To examine the effect of the treatments on asymmetry, we computed right/left ratios for each individual and all variables. The butterflies from which one hindwing disc had been removed tended to have a heavier forewing on the treated than on the untreated side (figure 2a). In contrast, the forewings of sham-treated individuals tended to be slightly heavier on the untreated side (figure 2b). There was no apparent asymmetry for the butterflies from which both hindwings had been removed, nor for forewing weight (figure 2c) nor for any of the other variables (results not shown), indicating that the removal of hindwing discs *per se* did not lead to asymmetry.

The principal test of our hypothesis is the difference in mean asymmetry between the treatment groups with removal of the hindwing disc on the left versus the right body side (table 2, upper part). This difference was highly significant for forewing weight, but weaker for the other measurements. The results for hindleg weight also supported the hypothesis (significant even after sequential Bonferroni adjustment for the tests in four variables), whereas the difference in asymmetries was not statistically significant for midleg weight. Surprisingly, forewing area showed a slight difference in the opposite direction.

The differences in mean asymmetry between the groups that received contralateral sham treatments were small (table 2, lower part). The only effect that was statistically significant (but non-significant if adjusted for multiple testing across variables) was for forewing weight, and it was opposite in direction to the difference expected for the disc removal treatments. Overall, the effect of injury, if any, would lead to a slight underestimate of the effect of hindwing disc removal.

4. DISCUSSION

Our results concur with those of a similar experiment by Nijhout & Emlen (1998), who found significant effects of the removal of one or both hindwing discs on the weights of the forewings, thorax and forelegs. The same pattern also holds for the weights of the mid- and hindlegs (table 1). The observation that removal of imaginal discs leads to increased growth in other structures further supports the hypothesis that growing imaginal discs compete for some resource.

Interestingly, whereas hindwing removal had a substantial effect on forewing weight, it had only a very weak and statistically non-significant effect on total forewing area (table 1). The asymmetry of forewing area even showed a slight response to disc removal in the direction opposite to that expected (table 2). This rules out the possibility that the increased allocation to the forewing on the treated side is adaptive compensation for the loss of hindwing area. The finding that the additional mass of the wing does not increase wing area is akin to results from *Drosophila*, where wing area is controlled by coordinated

Table 2. *Comparison of right/left ratios for measurements of wing and leg size*

(The upper part of the table shows the comparisons between the two experimental treatments, and the lower half the comparisons between the contralateral sham treatments. The tabled values are means (\pm standard error) of individual right/left ratios; comparisons were based on *t*-tests assuming equal variances, unless the *F*-test for unequal variances was significant (marked with a footnote symbol). Significance levels are not adjusted for multiple comparisons.)

	forewing weight (mg)	forewing area (mm ²)	midleg weight (mg)	hindleg weight (mg)
left hindwing disc removed	0.939 \pm 0.009	1.018 \pm 0.005	0.989 \pm 0.009	0.998 \pm 0.011
right hindwing disc removed	1.038 \pm 0.008	1.000 \pm 0.008	0.994 \pm 0.009	1.032 \pm 0.010
<i>t</i> (d.f.)	-8.16 (70)	2.00 (46) ^b	-0.47 (72)	-2.25 (72)
<i>p</i> (one-tailed) ^a	<0.0001	0.97	0.32	0.01
left side injured	1.010 \pm 0.007	1.014 \pm 0.005	1.009 \pm 0.009	1.004 \pm 0.005
right side injured	0.990 \pm 0.004	1.004 \pm 0.004	1.014 \pm 0.006	1.001 \pm 0.009
<i>t</i> (d.f.)	2.47 (56) ^b	1.86 (65)	-0.49 (65)	0.31 (53) ^b
<i>p</i> (two-tailed) ^a	0.02	0.07	0.62	0.76

^a For the hindwing disc removals, we used one-tailed tests because there was a clear expectation of the direction of differences. For the sham treatments, however, there was no such expectation, and we used two-tailed tests.

^b *t*-test for unequal variances.

variation in cell number and cell size (McCabe *et al.* 1997). Unfortunately, natural irregularities in the arrangement and loss of wing scales made scale counts too unreliable to estimate cell number in our study. In sum, the reallocation of resources by experimental manipulation of competition appears to elicit a specific response in weight and linear dimensions of imaginal structures. An increase in the amount of allocation to an imaginal disc does not simply lead to a proportional increase in all dimensions of the resulting imaginal structure, and thus suggests intrinsic regulation (e.g. Bryant & Schmidt 1990; McCabe *et al.* 1997).

Removal of the hindwing imaginal disc on one body side produced a considerable degree of asymmetry in other structures. The treatment produced an asymmetry in forewing weight of approximately 5% (average of left and right disc removals, table 2), which is nearly as much as the 7% overall difference in total weight of both forewings between the one-disc removal and control treatments (table 1). Because asymmetry refers to a comparison of individual wings (one forewing is 100%), but the overall effect is calculated from the total weight of both forewings (their sum is 100%), the asymmetry accounts for less than half of the additional mass acquired by the forewings. Similarly, for hindleg weight, the asymmetry between the treated and untreated sides is about 1.7%, whereas the increase in the average of both sides is approximately 2.7%. Overall, the increase in weight of structures is substantially greater on the body side from which the hindwing disc has been removed than on the untreated side. This asymmetry indicates that the interactions between growing structures are fairly localized.

The localization of interactions is also apparent from the decrease in the size of effects with increasing physical distance of the responding tissue from the location of the discs removed. Both the average weight increase and asymmetry in response to hindwing disc

removal are greatest for forewings, which develop from imaginal discs located immediately anterior of the hindwing discs. The hindlegs, located at a greater distance (although in the same segment), show an intermediate response, and the response is least for weight of the midlegs, which develop farthest from the hindwing discs. This gradual decay of the response with increasing distance is expected under the hypothesis of competition, and can provide information regarding the nature of the limiting resource.

A possible candidate for the limiting factor is the supply of oxygen. Yet, because the main tracheal trunks run along each body side and only much smaller transverse tracheae connect the left and right sides (Eaton 1988; Goyle 1990), one would expect the response to the removal of a hindwing disc to be confined almost completely to one side. The fact that the response was only partly asymmetric argues strongly against oxygen supply as the limiting factor.

The localized response is consistent, however, with the hypothesis of competition for a haemolymph-borne factor. As a preliminary study of haemolymph circulation, we injected fifth instar caterpillars with a vital stain (methylene blue) near the location of the hindwing imaginal discs (at the metathoracic subdorsal spine). This experiment showed that the haemolymph is effectively transported by peristaltic movements of the body wall. This flow is mostly along the sides of the caterpillar (but not near the dorsal or ventral midline) in an anterior-posterior direction, and to a lesser extent in the dorsal-ventral direction. Caterpillars dissected a short time after injection showed staining of the fat body and imaginal discs in the region surrounding the injection, and exclusively on that body side. Late dissections (*ca.* 2 h after injection) showed similar staining in all regions of both body sides. Caterpillars of *Precis coenia* lack dorsal and ventral diaphragms (as do other Lepidoptera (Eaton 1988)), and the heart and alary muscles are small and

deeply embedded between lobes of the fat body. It is therefore plausible that there is little directed haemolymph flow, but that the predominant mode of circulation is peristalsis by the body musculature as the caterpillar moves. Mixing of the haemolymph within and between body sides appears to occur gradually, and might therefore account for the graded response of forewings and legs to removal of hindwing discs.

This study suggests competition between growing structures as a possible mechanism for generating and regulating morphological asymmetries. Other mechanisms that have been demonstrated experimentally (Govind & Pearce 1992; Smith & Palmer 1994) or by comparison (Trinka *et al.* 1994) rely on differential use of structures on the two body sides. In contrast, competition for a limiting resource is a process intrinsic to the organism that can take place before structures are functional. Moreover, competition between growing organs does not require any mechanisms, such as an ability of the central nervous system to compare the sizes of organ primordia between body sides, for which empirical evidence is lacking. The only assumption is that growth is controlled by a limiting resource, which could be a growth factor or a nutrient. A range of morphological outcomes can be produced by varying the uptake of the resource and the rate of haemolymph circulation. If circulation is slow, faster growth of a structure will lead to local depletion and tend to limit further growth, and thus can exert a stabilizing influence by local negative feedback. This may be the normal situation in *Precis*. In contrast, rapid mixing will distribute the effects throughout the organism; if combined with an increase in the cell-specific rate of resource uptake and growth on one side, this will lead to asymmetric growth of one body side at the expense of the other. Feedback is thus somewhat different from the model of Graham *et al.* (1993) in that it is not limited to corresponding organs on the two body sides, but can simultaneously affect multiple growing structures (Nijhout & Wheeler 1996; Nijhout & Emlen 1998). We expect that further study of these interactions will lead to novel insights into the mechanisms underlying developmental stability.

We thank Laura Grunert for maintenance of the buckeye colony, and Douglas Emlen, Jeff Marcus, Armin Moczek, Rich Palmer, David Stern, Tomalei Vess and two anonymous referees for discussion and comments on earlier versions of the manuscript. Financial support was provided by a fellowship from the Swiss National Science Foundation to C.P.K.

REFERENCES

- Batterham, P., Davies, A. G., Game, A. Y. & McKenzie, J. A. 1996 Asymmetry—where evolutionary and developmental genetics meet. *BioEssays* **18**, 841–845.
- Bryant, P. J. & Schmidt, O. 1990 The genetic control of cell proliferation in *Drosophila* imaginal discs. *J. Cell Sci.* **13** (Suppl.), 169–189.
- Bryant, P. J. & Simpson, P. 1984 Intrinsic and extrinsic control of growth in developing organs. *Q. Rev. Biol.* **59**, 387–415.
- Chippindale, A. K. & Palmer, A. R. 1993 Persistence of subtle departures from symmetry over multiple molts in individual brachyuran crabs: relevance to developmental stability. *Genetica* **89**, 185–199.
- Davies, A. G., Game, A. Y., Chen, Z., Williams, T. C., Goodall, S., Yen, J. L., McKenzie, J. A. & Batterham, P. 1996 *Scalloped wings* is the *Lucilia cuprina* *Notch* homologue and a candidate for the *Modifier* of fitness and asymmetry if diazinone resistance. *Genetics* **143**, 1321–1337.
- Eaton, J. L. 1988 *Lepidopteran anatomy*. New York: Wiley.
- Govind, C. K. & Pearce, J. 1992 Mechanoreceptors and minimal reflex activity determining claw laterality in developing lobsters. *J. Exp. Biol.* **171**, 149–162.
- Goyle, S. 1990 *Anatomy of the common lemon butterfly* *Papilio demoleus demoleus* (L.). New Delhi: Today & Tomorrow's Printers and Publishers.
- Graham, J. H., Freeman, D. C. & Emlen, J. M. 1993 Antisymmetry, directional asymmetry, and dynamic morphogenesis. *Genetica* **89**, 121–137.
- Kawano, K. 1997 Cost of evolving exaggerated mandibles in stag beetles (Coleoptera: Lucanidae). *Ann. Ent. Soc. Am.* **90**, 453–461.
- Leamy, L. J., Routman, E. J. & Cheverud, J. M. 1997 A search for quantitative trait loci affecting asymmetry of mandibular characters in mice. *Evolution* **51**, 957–969.
- Markow, T. A. 1995 Evolutionary ecology and developmental instability. *A. Rev. Entomol.* **40**, 105–120.
- McCabe, J., French, V. & Partridge, L. 1997 Joint regulation of cell size and cell number in the wing blade of *Drosophila melanogaster*. *Genet. Res. Camb.* **69**, 61–68.
- Møller, A. P. 1996 Development of fluctuating asymmetry in tail feathers of the barn swallow *Hirundo rustica*. *J. Evol. Biol.* **9**, 677–694.
- Møller, A. P. & Swaddle, J. P. 1997 *Asymmetry, developmental stability, and evolution*. Oxford University Press.
- Nijhout, H. F. & Emlen, D. J. 1998 Competition among body parts in the development and evolution of insect morphology. *Proc. Natn. Acad. Sci. USA* **95**, 3685–3689.
- Nijhout, H. F. & Wheeler, D. E. 1996 Growth models of complex allometries in holometabolous insects. *Am. Nat.* **148**, 40–56.
- Palmer, A. R. & Strobeck, C. 1986 Fluctuating asymmetry: measurement, analysis, patterns. *A. Rev. Ecol. Syst.* **17**, 391–421.
- Smith, L. D. & Palmer, A. R. 1994 Effects of manipulated diet on size and performance of brachyuran crab claws. *Science* **264**, 710–712.
- Swaddle, J. P. 1997 On the heritability of developmental stability. *J. Evol. Biol.* **10**, 57–61.
- Swaddle, J. P. & Witter, M. S. 1997 On the ontogeny of developmental stability in a stabilized trait. *Proc. R. Soc. Lond. B* **264**, 329–334.
- Trinka, E., Churchill, S. E. & Ruff, C. B. 1994 Postcranial robusticity in *Homo*. II. Humeral bilateral asymmetry and bone plasticity. *Am. J. Phys. Anthropol.* **93**, 1–34.

