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Are entrenched characters developmentally constrained? Creating biramous limbs in an insect

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SUMMARY Are evolutionarily entrenched phenotypes highly constrained developmentally? We explored this question in the case of the uniramous appendages of fruit flies. We created bi- and polyramous antenna/leg combinations in four different genotypes. Each genotype consisted of two relevant mutations. We suggest that not all entrenched characters are strongly constrained by developmental processes and that there exists sufficient natural genetic variation to alter highly conserved phenotypes.

INTRODUCTION

Darwin's concept of "descent with modification" as an explanation for both the similarities and differences between taxa is now being explored by biologists from a variety of subdisciplines. Modern developmental biology has recently joined comparative and experimental embryology, paleontology, systematics, and genetics in trying to unravel the evolutionary history of organisms. With the tools of developmental genetics, a vast array of developmental homologies has been established based upon comparative gene expression profiles (Gerhart and Kirschner 1997). Currently, developmental biologists examining evolutionary questions have focused their attention on describing the evolutionary history of development, as compared with asking whether and how developmental processes shape evolutionary patterns. In this paper, we take an experimental approach to exploring one frequently encountered feature in evolution: a character may be highly entrenched in one taxon (such as segment numbers in insects) (Brusca and Brusca 1990) but not in others (segment numbers in myriapods, crustaceans) (Arthur 1999). What are the developmental and genetic correlates of entrenchment? Two contrasting explanations for entrenchment may be advanced: character states are maintained by natural selection or they are the result of developmental constraints that channel the phenotype in a particular direction. These alternative explanations are by no means mutually exclusive, and in fact they could both be operating in maintaining a particular phenotype. While overwhelming evidence for the existence of natural selection has been accumulating for over a century, the existence of developmental constraints is not so well established (Hall 1996; Kauffman 1983). In fact, the concept of developmental constraints, like most basic concepts, is difficult to define in a satisfactory manner (Maynard Smith et al. 1985). It is useful to distinguish between absolute (qualitative) constraints and those of a quantitative nature. What we are calling "qualitative constraints" are those morphologies that cannot evolve as a result of the developmental system of ancestral organisms. "Quantitative constraints," on the other hand, do not pose an absolute restriction on possible morphologies, but bias the direction and rate of phenotypic evolution.

With respect to morphogenesis, a theoretical argument suggests that such constraints on form are not absolute as a few (overlapping) cell behaviors (division, death, growth, shape change, movement, matrix secretion) account for all multicellular morphogenesis. In principle, any morphology should be obtainable by manipulating these cell behaviors in time and space (Larsen 1997). To explicitly test for developmental constraints in a particular entrenched character we used a "designer organism" approach. By successfully altering such a character in a prescribed way we are in essence testing the hypothesis that there are no absolute morphological developmental constraints for that character.

As our first test, we have produced a biramous insect appendage. The insects and myriapods are known as "uniramous arthropods" because their trunk appendages have a single proximal distal axis (Meglitsch and Schram 1991). Crustacean appendages, on the other hand, may have two or more branches emanating from a common appendage segment (Williams and Muller 1996). These two branches (rami) are sometimes structurally and functionally differentiated from one another,

for instance in the walking ramus and gill ramus combination (Brusca and Brusca 1990). Are insects capable of evolving bi- or polyramous appendages? Are there constraints preventing them from doing so? We conceived a plan to achieve a biramous antenna/leg appendage with a claw at the end of the leglike appendage and an arista at the terminus of the antenna-like branch. We used combinations of two types of mutations to achieve this morphology in fruit flies. One type of mutation produced a duplicated antenna and the other type transformed the antenna into a leglike structure.

METHODS

Fly stocks

The following is a list of genotypes used in this study (Flybase, at www.flybase.indiana.edu):

white (*w*); obake (*obk*)
obk, hedgehog β-galactosidase (*hh-LacZ*)
w; *obk*; spineless aristapedia (*ss*[*a*])
w; *obk/obk* Sternopleural (*Sp*); Antennapedia [73b] (*Antp*[*73b*]), *homothorax* [*1422-4*]*TM3*
w; *obk/obk* (*Sp*); Antennapedia (*Antp*[*Ns*]), *hth* [*1422-4*]*TM3*
w; *obk*; Heat Shock Antennapedia (*HSAntp*)

Crosses

To combine *obk* with third chromosome alleles, standard crosses were made with the dominant marker, homozygous lethal balancer stock with markers *w; CyO/Sp; D/ TM3,Sb*. As noted, some stocks contained a floating *Sp* allele and the *Antp* alleles were combined with the P *hth* [*1422-4*] and balanced over either *TM3 Sb* or *TM3 Ser GFP*. *hth* [*1422-4*] expresses β galactosidase in a pattern consistent with homothorax protein during larval development (Salzberg et al. 1997). *Homothorax* is useful to monitor downstream effects of *Antp* but biramous structures were also found when it was not included in *obk, Antp*[*73b*] genotypes. The *hth*[*1422-4*] stock was supplied by A. Salzberg. The second chromosome deficiency kit, *b, cn, bw* mapping line, *engrailed*[*1*] (*en*[*1*]), *en*[*7*], and *en* deficiencies were all courtesy of the Bloomington stock center. The *en*[*E*]*/CyO* allele was provided by Dr. Carol Schwartz, and the *en*[2] allele by Dr. Thomas Kornberg. The laboratory of M. Scott provided the *HSAntp* stock. Gene annotation numbers are according to Gadfly (http://hedgehog.lbl.gov:8001/cgi-bin/annot/query).

Immunohistochemistry and β-galacatosidase staining protocols

Antibody staining was based upon standard protocols (White 1998). In brief, imaginal discs were fixed 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), then washed in PBS with 0.19% Triton-X, blocked, and incubated overnight with the 4D9 anti-engrailed mouse monoclonal antibody (Patel et al. 1989) obtained from the Developmental Studies Hybridoma Bank in Iowa. 4D9 was used at a concentration of 1:10. For the secondary antibody, a goat anti-mouse conjugated to horseradish peroxidase (preabsorbed) was used at a concentration of 1:400 and incubated overnight. Following washes, the tissue was incubated in diaminobenzimide (DAB):PBS (1:4) for 15 minutes, either with or without NiCl, and then 0.0015% hydrogen peroxide was added to allow the development of the stain. Disks were mounted in 70% glycerol in PBS. β-galacatosidase staining and scanning electron microscopy were performed as described in Scanga et al. (1995).

Antenna duplication and transformation

obk was used to induce duplicated antennal primordia. To transform one antenna duplicate into a leg, we experimented with both mutations and transgenes. Among mutations we used dominant alleles at the Antennapedia locus, *Antp*[*73b*] and *Antp*[*Ns*] and the recessive *ss*[*a*] allele. The Antp alleles ectopically express Antennapedia protein (ANTP) in the antenna disc, producing antenna to second leg transformations, which often include distal tarsal structures as well as proximal structures like the femur. In contrast, *ss*[*a*] is a loss-of-function allele; the wild-type function promotes antennal identity (Duncan et al. 1998). The mutant transformation consists only of distal leg (tarsal) structures. We also used the *HSAntp* transgene in combination with *obk*. In this case, we heat-shocked during the third larval instar at either 56 or 60 h after hatch and tested both 10 and 40 minute durations at 37°C to determine the temporal sensitivity to biramous transformation. Unless otherwise stated, stocks were raised on a corn meal/sucrose/yeast medium at room temperature.

RESULTS AND DISCUSSION

Genetic analysis of obake

obk is a mutation that was discovered in a P-element mutagenesis screen for modifiers of the Bar phenotype. The *obk* phenotype has several distinct aspects. The most prominent phenotypic effect observed is the mirror image duplication (compare Figs. 1A and 1B) and sometimes triplication of the antenna. There is a great deal of variation in these phenotypes, ranging from duplication of distal elements of the antenna (arista), to complete duplication of the entire antenna. In addition to duplication of antennae, the *obk* phenotype may include duplicated or absent maxillary structures, reduced eyes, and abnormal bristle patterns on the face. Duplications of antennal structures and maxillary palps may occur on the same side of the head.

The highest frequencies of duplications (20–30%) occur in young cultures. We assume that the reduction in frequency of duplications as cultures age is the result of deteriorating nutritional and environmental factors that either reduce the ability of duplicates to grow or reduce the viability of those larvae with duplicates.

Genetic mapping of *obk*

obk was localized to the second chromosome, 2.5 map units distal to *cinnibar* (*cn*) by meiotic mapping. We attempted to map the locus responsible more precisely by deficiency mapping. Using the second chromosome deficiency kit, we crossed *w; obk* females to males of each deficiency. How-

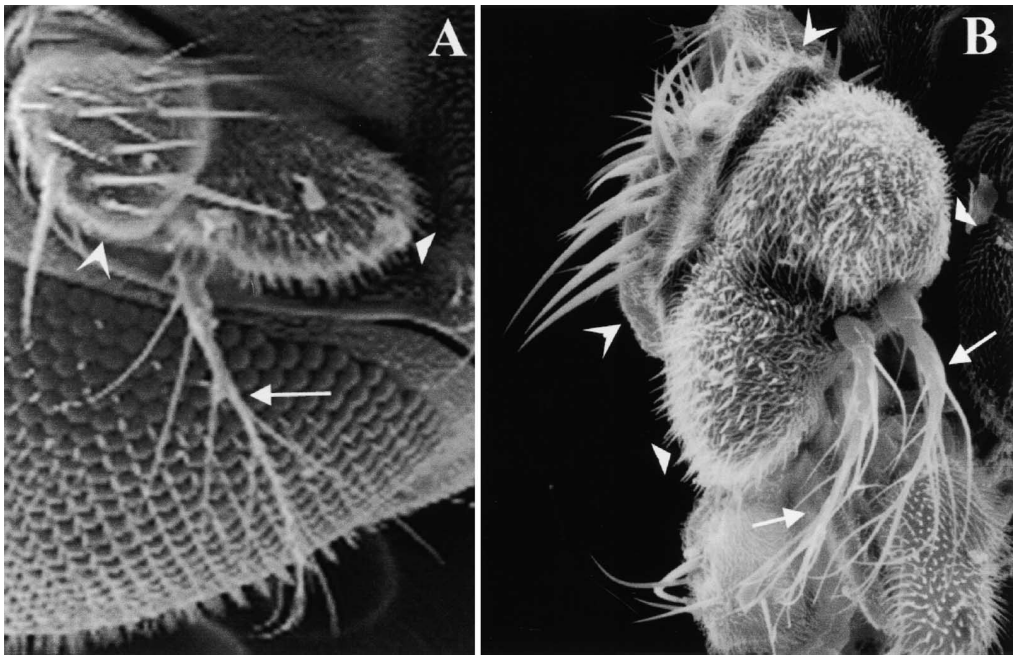

Fig. 1. Comparison of wild-type and obake antennae. (A) The wild-type antenna has a single distal arista (arrow) developing from the third antennal segment (flat arrow head), which is densely covered with trichomes. In contrast, the more proximal, second antennal segment (concave arrowhead) has only large bristles. (B) Antennal duplication resulting from the obake mutation showing a duplicated second (concave arrowhead), third (flat arrowhead), and sixth/arista (arrow) antennal segments in mirror image symmetry.

ever, the fact that *obk* shows variable expressivity and penetrance resulted in some ambiguities. While several deficiencies showed some eye phenotypes when transheterozygous to *obk* (Df(C2R)*Pcl11B*, Df(2R)*vg*135, Df(2L)*dp-79b*), only Df(2R)*en-A*/CyO and Df(2R)*en30*/Sm5 failed to complement the antennal duplication (although the latter produced only one individual, out of about 50, with duplications). This evidence suggests that *obk* maps near the *en/inv* gene. We have observed antennal duplications in some *obk/CyO* flies but have not yet determined whether this is due to a factor on the *CyO* balancer or other genetic background effects. We do not believe *obk* is dominant as *obk/obk*[+] heterozygotes produce no evidence of antennal duplicates.

To further explore the interaction of *obk* with genes in the region of the *en* locus, we crossed *w; obk* females to a number of local deficiencies and mutations of that region. The results from this complementation analysis are summarized in Table 1. Four of the five *en* deficiencies failed to complement *obk* for the antennal duplications, and all produced *en*-like wing phenotypes as did the *en*[*E*] allele (which is a local deletion spanning all of *en* and part of the *inv* open reading frame) (Fig. 2). Only mutations in the open reading frame of *en* complemented both the antennal and wing phenotypes. *obk* homozygotes revealed no wing venation abnormalities. Although duplicated antennae are not phenotypes associated with *en* homozygotes, they are found in some heteroallelic combinations such as *en*[1]/*en*[2] (Morata and Lawrence 1979). The data taken together suggest that *obk* maps to the 48A1-B1 cytological region and is possibly an allele of *en, inv,* or a nearby gene that interacts with *en/inv*. Other genes in this region include a putative transcription factor binding protein (CG10897), a putative DNA binding protein (CG9006), a cell adhesion molecule (CG9005), and an acyl-coA dehydrogenase.

Gene expression associated with secondary morphogenetic fields

We examined the expression of several candidate genes known for mediating patterning in imaginal discs. Figure 3 shows the expression of EN/INV in wild-type and *obk* discs. EN/INV expression can be seen in both the normal and duplicate fields in the posterior compartment of the disc. X-Gal staining of *obk hh-lacZ* shows a duplicated expression pattern similar to that of EN/INV. All other enhancer traps examined, *dll*, *wg*, and *hth*, exhibit duplicated expression patterns (not shown). These results differ from those reported

Table 1: Complementation analysis of *obk* with deficiencies and mutations of engrailed.

Genotype	Antennal duplications	Wing venation defects	Ectopic "eye" bristles
Df(2R)*en*28/ *obk*	–	+	+
Df(2R)*en-SFX/ obk*	+	+	+
Df(2R)*en-A*/ *obk*	+	+	+
Df(2R)*en-B*/ *obk*	+	+	+
Df(2R)*en*30/ *obk*	+[1]	+	+
en^{E}/ obk	–	+	–
en^{7}/ *obk*	–	–	–
en^{2}/*obk*	–	–	–
en^{1}/ *obk*	–	–	–

+, production of phenotypic defect (failure to complement). –, no phenotypic defect observed (mutation complemented obk).
[1] Only a single individual was observed with antennal duplications.

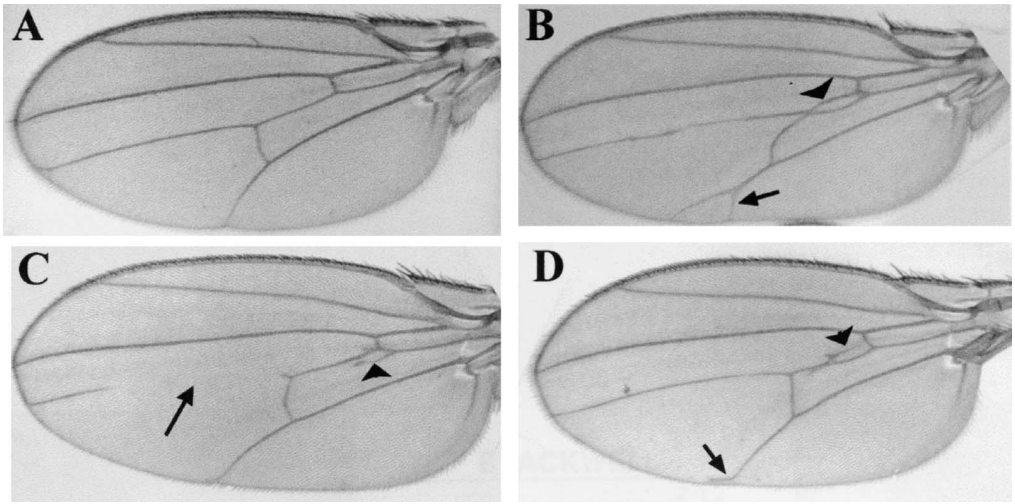

Fig. 2. Wing phenotypes of *obk* crossed with various chromosomal deficiencies of the engrailed region. (A) Wing with wild-type venation patterns. (B) *obk* crossed with the *en*28 deficiency. Arrow points to a local duplication of the fifth (L5) vein. (C) *obk* crossed to *en*B deficiency. Loss of a portion of the medial region of L4 (arrow). (D) *obk* crossed to *en*[E] deficiency. Small distal extension of L5 (arrow). Arrowheads (in B, C, and D) show the duplication of a part of L3 near the anterior cross-vein at the border of the posterior and anterior compartments. Anterior is toward the top, distal to the left. The length of each wing is approximately 3 mm (from hinge to distal tip of L3).

for *en* alleles (Condie and Brower 1989), where it was suggested that *en*-induced duplications were mirror images of anterior pattern at the expense of posterior regions (Morata et al. 1983). Our results suggest that *obk* produces a secondary posterior domain (Fig. 3), which is sufficient to induce expression of both posterior and anterior patterning genes (Tabata et al. 1995). Thus, if *obk* is indeed an allele of *en* or *inv*, it may be a neomorphic mutation affecting the enhancer.

To determine whether the duplicated disc arises in a fixed position relative to the eye disc, we looked at *obk, hh lacZ* discs in early third instar. We found that in younger larvae with duplicates, the smaller duplicate was always closer to the eye. By the end of the third larval instar, the duplicates were comparable in size. These findings suggest that in *obk* discs the antennal field duplicates at some time during larval development rather than at the time of the embryonic formation of the presumptive antennal disc. This is consistent with previous observations that the antenna and eye discs show relatively late periods of final determination (Morata and Lawrence 1979).

Producing the biramous, antenna/leg phenotype

Two approaches to designing a biramous structure come to mind: partial fusion of two rudiments or production of duplicates from a single rudiment. We used the latter approach because of the existence of several different mutations that cause antenna duplications. More interesting than just producing a biramous structure via duplication is producing a morphological novelty in which the appendage assumes new form, in our case, producing antenna/leg combinations. In principle, any mutation that produces duplicated antennal discs could have been used. We only used one duplication generating mutation (*obk*), because others such as *su*(*f*)[*12*] and *ex*[*br*] exhibited low penetrance in our hands.

To achieve bi- or polyramous limbs, we crossed mutations capable of inducing antenna-to-leg transformations into a homozygous *obk* background. The *obk; Antp*[*73b*]*, hth* [*1422-4*] genotype produced the most satisfactory antenna/leg combinations. Figures 4A and 4B provide a near-ideal realization of the phenotype we had in mind at the outset. The antennal leg has both proximal and distal structures including a claw, and a partial A3 segment bears an identifiable arista, which is fairly normal. There are, however, leglike bristles on the A3 remnant. In Fig. 4C a partial head is shown in which the details of the antennal duplication are apparent and the relationship of the biramous appendage to the eye is seen.

obk, Antp[*Ns*] stocks also produced antenna/leg combinations. The phenotypes tended to be complicated and less easily interpreted than in other genetic combinations. Sometimes the antennal leg is duplicated and a misshapen arista is found at some distance. We have found up to five legs on a single fly head. Table 2 shows frequency data for bi- or polyramous transformations at 25°C and room temperature for stocks with different *Antp* alleles. Eclosed and pharate adults that died in the pupal case are included in these results. For *Antp*[*73b*] frequencies were higher at room temperature than at 25°C, whereas temperature had little effect on the frequency of transformations in *Antp*[*Ns*] despite the fact that this allele is temperature-sensitive (Jowett and Sang 1979). Males showed no transformation in *obk; Antp*[*Ns*]*,hth* [*1422-4*], and this stock

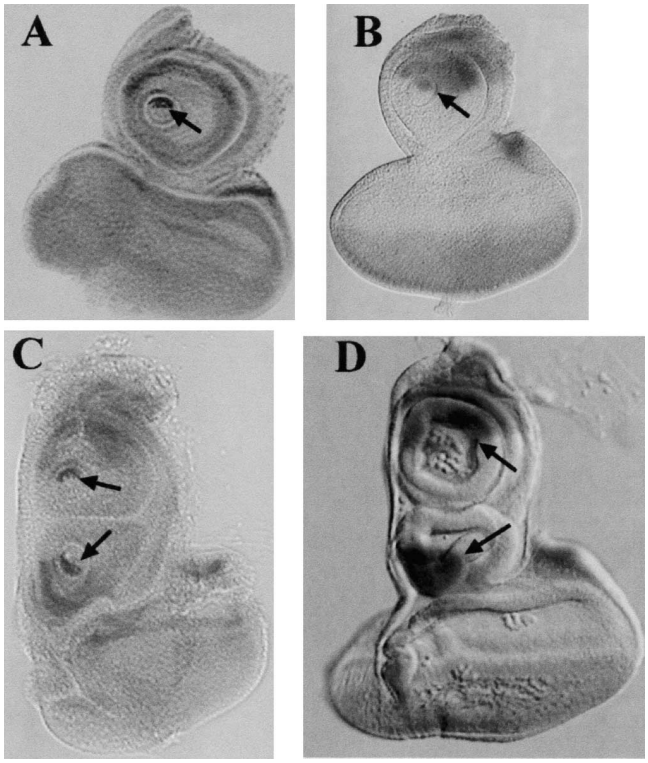

Fig. 3. Expression of EN and hedgehog in *obk* and wild-type eye-antenna imaginal discs. (A and C) expression of EN, monitored using the 4D9 antibody in wild-type (A) and obk (C) discs. Arrow points toward EN-expressing cells in the posterior compartment of the antenna disc. The circular region is a fold in the disc epithelium, corresponding to presumptive distal antenna. In (C) the lower arrow points to the duplicated region of EN-expressing cells, corresponding to a new posterior region of antenna. A second circular fold in the epithelium can also be seen. (B and D) expression of *hh*, monitored using a *hh-LacZ* reporter gene in wild-type (B) and *obk* (D) discs. Arrows point to regions of hedgehog-expressing cells in the antenna disc. As in (C), (D) shows a duplicated pattern of expression corresponding to the posterior region of the antenna disc. The diameter of the antennal disc (left to right) is approximately 200 μm.

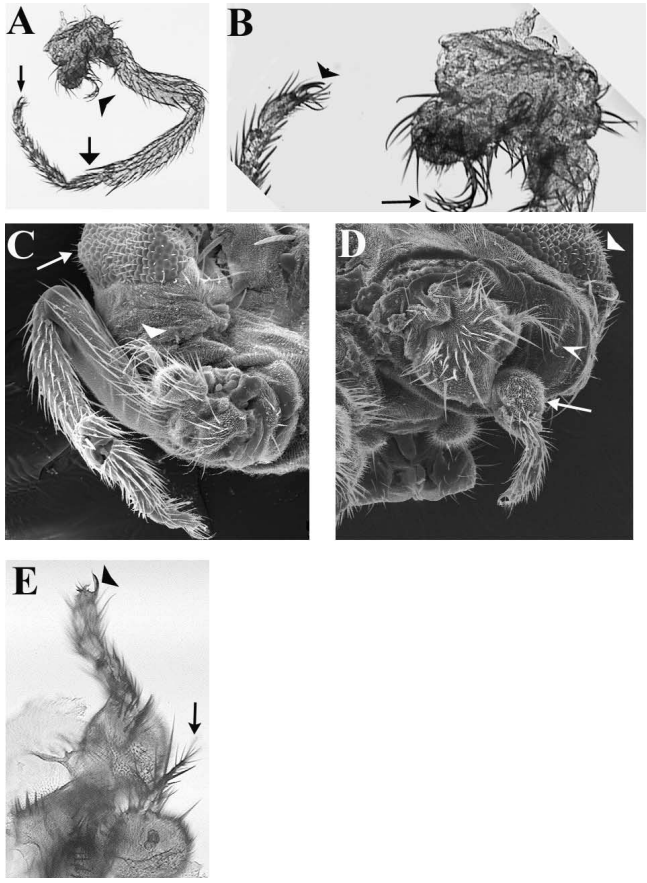

Fig. 4. Biramous antennal/leg combinations. (A) Antenna/leg combination of the *w; obk; Antp*[*73b*],*hth*[*1422-4*] genotype. The transformed antennal leg contains both proximal and distal elements of a second leg including a claw on the tip of the tarsus (arrow) and an apical bristle (wide arrow). The arista emanates from a partial third antennal segment that has long leglike bristles suggesting partial transformation to leg (arrowhead). (B) Enlargement of appendage in (A) showing details of the arista (arrow) and claw regions (arrowhead). (C) A scanning electron micrograph of an *w; obk; Antp*[*73b*],*hth*[*1422-4*] head region to show additional details of the duplicated and transformed regions as well as the relationship of these structures to the eye (arrow). An arrowhead points to the arista. (D) A scanning electron micrograph of a *w; obk; ss*[*a*] head region with eye in the upper right (arrowhead). The tarsal segments derive from a fairly normal third antennal segment (arrow) that shows few signs of transformation while the somewhat thickened arista (concave arrowhead) emerges from tissue beneath a duplicated second antennal segment, which has not bifurcated to the extent found in (C). (E) Antenna/leg combination of the *w; obk; ss*[*a*] genotype. The *ss*[*a*] transformation consists primarily of distal leg structures including the claw (arrow). The arista (arrowhead) is somewhat thickened and the third antennal segment shows only a few leglike bristles.

also showed a higher frequency of antenna duplicates and/or antennal leg duplicates compared to *obk; Antp*[*73b*], *hth* [*1422-4*]. These results suggest that both genotype and environment are important in producing variation in the biramous phenotype. We do not understand why the *obk; Antp*[*73b*] duplicates almost always produce antenna/leg combinations compared to *obk; Antp*[*Ns*]. This variation may be related to differences in developmental timing in the duplicate antennal fields, which as noted above grow at different rates.

Figures 4D and 4E show biramous appendages produced by *obk; ss*[*a*]. Like the *ss*[*a*] phenotype in the absence of *obk* (not shown), the antennal leg included tarsal but not proximal structures. Claws were rarely found on the distal tarsal tips. As with *Antp*[*Ns*], duplicated antennal legs were frequently encountered. Antenna/leg combinations were found in about 20% of flies in one stock selected for polyramous antennae.

The *obk; HS-Antp* genotype produced bi- or polyramous appendages with both antenna and leg combinations after heat shocks of 40 min duration at 37°C at either 56 or 60 h

Table 2. Penetrance of the biramous phenotypes.

Genotype	Sex	Temp (degrees C)	*n*	% Antenna/ leg biramous	95% Confidence interval	% Antenna duplicate or leg duplicate	95% Confidence interval
w; obk; Antp[73b], hth[1422-4] /Tm3 Sb (note about 1/3 are obk/obk, Sp)	M & F	25	172	5.8	2.3–9.3	0	—
	F		96	4.2	0.2–8.2	0	—
	M		66	9.1	2.2–16.0	0	—
w; obk; Antp[73b], hth[1422-4]/Tm3 Sb	M & F	RT	114	17.5	10.5–24.5	0.9	0–2.6
	F		67	22.4	12.4–32.4	1.5	0–4.4
	M		47	10.6	1.8–19.4	0	—
w; obk; Antp[Ns], hth[1422-4]/Tm3 Sb	M & F	25	144	4.2	1.0–7.4	6.9	2.8–11.0
	F		93	6.5	1.5–11.5	10.8	4.4–17.2
	M		51	0	—	0	—
w; obk; Antp[Ns], hth[1422-4]/Tm3 Sb	M & F	RT	223	4	1.4–6.6	5	2.1–7.9
	F		116	7.8	2.9–12.7	9.5	4.2–14.8
	M		107	0	—	0	—

RT = room temperature, approximately 22°C.

after hatch. These resembled *obk; ss*[*a*] flies in that the leg-like tissue contained primarily tarsal structures with or without claws. We conclude that the *obk*-associated duplication had occurred by 56 h and that the competence to respond to ANTP protein differed between some of the duplicates such that only one responded to ANTP by transforming to leg.

What does the insect polyramous phenotype tell us?

In sum, these results show that bi- or polyramous structures can be produced in a unirame arthropod and that this phenotype can be generated in several different genotypes. It may seem surprising that only two gene changes were required to produce a bi- or polyramous phenotype in a taxon with the highly entrenched uniramous phenotype. However, the mutations used were of large effect and are unlikely to be found in nature. In particular, the ability of *obk* to duplicate large portions of the antenna imaginal disc morphogenetic field was crucial to our success. The characteristics of morphogenetic fields (gradient of activity, size invariance) provide a highly evolvable tissue level "module" (Gilbert et al. 1996). While the cellular and molecular mechanisms that give rise to field properties are still enigmatic, we have long known how to induce new fields through surgical bifurcation (Bryant 1971), genetically induced cell death (Clark and Russell 1977), and mis-expression of genes involved in pattern formation (Basler and Struhl 1994; Diaz-Benjumea et al. 1994). Thus we feel that there are a variety of developmental mechanisms potentially available in nature to produce local duplications. Having failed to detect cell death in third instar *obk* antenna imaginal discs, we suspect that *obk* does not use cell death in creating a new morphogenetic field. Rather, its interaction with alleles of the *en* locus suggest that mis-expression of genes involved in pattern formation is more likely.

Homeotic mutations were the other type of mutation used. These mutations also have global coordinating properties in that they integrate several aspects of changes in organ determination: morphogenesis (antenna to leg transformation), cell differentiation (antenna- or leg-specific bristles), and cell patterning (location and arrangement of bristle arrays). Although our purpose has been to explore the constraints on morphology, it is well to point out that the phenotypes of the mutations used here can be achieved by mutations at a number of different loci and are influenced by modifiers elsewhere in the genome (Jowett and Sang 1979). Therefore, it is possible to imagine scenarios in which genetic variations for a polyramous fly appendage could arise in natural populations (Waddington 1961). Indeed, Hardy (as shown in [Ashburner 1989)] found several Hawaiian Drosophilid species with bifurcating tarsi, changes that might presage the evolution of different functions for each branch as in crustacean leg/gill combinations. In fact within the *Plecoptera* (stoneflies), there are some species in which each coxa bears a gill. These gills are apparently not homologous to those of crustaceans (Zwick 2000). We are not suggesting that either the branching appendages of extant insects or the biramous limbs of crustacea arose through our method of gene-controlled duplication and modification. Rather, just as Gibson and Hogness (1996) found natural polymorphisms in the *Ubx* gene responsible for variation in the ability to transform haltere to wing, it is possible that natural genetic variation exists to produce branched structures in insects. Indeed, the maxillae of insects appear to be branched (for example the maxillary palpus and lacinia in *Drosophila*), and because the patterning mechanisms of trunk and mouthpart appendages share some similarity (Abzhanov and Kaufman 1999), the common genetic mechanisms may provide a source of developmental variation for producing biramous appendages from uniramous ones.

If the developmental constraints preventing polyramy are fairly weak, are there factors (other than selection) that reduce the likelihood of the evolution of such limbs? We note that although the frequency of induced polyramy varies with genotype and environment, the maximum frequencies of about 20% are surprisingly high. What is apparent is that the phenotypes are variable within and between genotypes. The implication here is that it is relatively easy to produce a polyramous structure with aspects of two different types of appendages; it is harder to achieve a consistent phenotype. Relatively uniform phenotypes are the basis for defining a wild-type phenotype and ultimately for our ability to identify organisms on the basis of their morphology. Thus while our evidence does not suggest any absolute developmental constraint for insects to produce biramous appendages, the inability of the organisms to produce a well-canalized (stable) phenotype from generation to generation may itself be considered a type of constraint. The problem with Goldschmidt's "hopeful monsters" may be that even assuming a selective advantage, low penetrance would reduce the probability of accumulating modifiers that stabilized the novel phenotype.

The designer organism research program

A designer organism research program should be able to illuminate several other developmental issues of evolutionary significance. For example, there has been recent interest in defining character states that are important in constructing phylogenetic trees and in defining functionally relevant characters in testing biological models (Wagner and Laublicher 2000). One kind of measure is the response of developing systems to environmental perturbation (H. Larsson, personal communication). In this approach one may use experimental intervention to judge the ease or difficulty of disrupting the normal phenotype. Our genetic "designer organism" approach uses genes rather than exogenous treatments to change morphology in defined ways (e.g., size or shape modifications). By using genes that perturb different phenotypic characters in similar ways, we can compare the relative stability of the characters.

The designer organism approach has a venerable history. Experimental embryologists have created "monsters" by altering limb numbers and structure in vertebrates utilizing surgical and chemical (for example, retinoic acid) modifications. Ectopic expression of ANTP (Schneuwly et al. 1987) or eyeless protein (Quiring et al. 1994) using transgenes in flies provides genetic manipulations within the designer organism framework. Other excellent examples of how contemporary genetic manipulations can provide strong inference concerning evolutionary changes are seen in work of Wimmer et al. (2000), showing that bicoid in flies can be replaced with hunchback (hb) as well as the older work showing that if both nanos and hb are eliminated a normal posterior region can be obtained (Irish et al. 1989). Our work and the above examples may be distinguished from the examination of mutations with apparent atavistic phenotypes (Lewis 1978; Palsson and Gibson 2000). In designing phenotypes, we are testing explicit hypotheses about developmental mechanisms, as opposed to reconstructing possible evolutionary transitions. We believe that the designer organism research program can creatively complement more traditional lines of historical investigation and should become better integrated as an experimental approach for exploring the patterns and processes responsible for biodiversity.

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REFERENCES

Abzhanov, A., and Kaufman, T. 1999. Novel regulation of the homeotic gene *Scr* associated with a crustacean leg-to-maixilliped transformation. *Development* 126: 1121–1128

Arthur, W. 1999. Variable segment number in centipedes: population genetics meets evolutionary developmental biology. *Evol. & Dev.* 1: 62–69.

Ashburner, M. 1989. *Drosophila, A Laboratory Handbook*. Cold Spring Harbor Press, Cold Spring Harbor.

Basler, K., and Struhl, G. 1994. Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* 368: 208–214.

Brusca, R. C., and Brusca, G. J. 1990. *Invertebrates*. Sinauer Associates, Inc., Sunderland, Massachusetts.

Bryant, P. J. 1971. Regeneration and duplication following operations *in situ* on the imaginal discs of *Drosophila melanogaster*. *Dev. Biol.* 26: 606–615.

Clark, W. C., and Russell, M. A. 1977. The correlation of lysozymal activity and adult phenotype in a cell-lethal mutant of *Drosophila*. *Dev. Biol.* 57: 160–173.

Condie, J., and Brower, D. 1989. Allelic interactions at the engrailed locus of Drosophila: engrailed protein expression in imaginal discs. *Dev. Biol.* 135: 31–42.

Diaz-Benjumea, F., Cohen, B., and Cohen, S. 1994. Cell interactions between compartments establishing the proximal-distal axis of Drosophila legs. *Nature* 372: 175–178.

Duncan, D. M., Burgess, E .A., and Duncan, I. 1998. Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes Dev.* 12: 1290–1303.

Gerhart, J., and Kirschner, M. 1997. *Cells, Embryos, and Evolution: Toward a Cellular and Developmental Understanding of Phenotypic Variation and Evolutionary Adaptability*. Blackwell, Abingdon.

Gibson, G., and Hogness, D. 1996. Effect of polymorphism in the *Drosophila* regulatory gene *Ultrabithorax* on homeotic stability. *Science* 271: 200–203.

Gilbert, S. F., Opitz, J. M., and Raff, R. A. 1996. Resynthesizing evolutionary and developmental biology. *Dev. Biol.* 173: 357–372.

Hall, B. K. (1996) *Bauplane, Phylotypic Stages, and Constraint: Why Are There So Few Types of Animals?* Evolutionary Biology Vol. 29. Plenum Press, New York.

Irish, V., Lehmann, R., and Akam, M. 1989. The *Drosophila* posterior-group gene *nanos* functions by repressing *hunchback* activity. *Nature* 338: 646–648.

Jowett, T., and Sang, J. H. 1979. Nutritional regulation of antennal/leg homoeotic mutants in Drosophila melanogaster. *Genet. Res.* 34: 143–161.

Kauffman, S. A. 1983. Developmental constraints: internal factors in evolution. In B. C. Goodwin, N. Holder, C. C. Wylie, et al. (eds.). *Development and Evolution.* Symposia of the British Society for Developmental Biology Vol. 6. Cambridge University Press, Cambridge, pp. 195–225.

Larsen, E. W. 1997. Evolution of development: the shuffling of ancient modules by ubiquitous bureaucracies. In C. Lumsden, W. A. Brandts, L. E. H. Trainor, et al. (eds.). *Physical Theory in Biology*. World Science, Singapore, pp. 431–441.

Lewis, E. B. 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* 276: 565–570.

Maynard Smith, J., Burian, R., Kauffman, S., et al. 1985. Developmental constraints and evolution. *The Quarterly Review of Biology* 60: 265–287.

Meglitsch, P., and Schram, F. R. 1991. *Invertebrate Zoology.* Oxford University Press, New York.

Morata, G., Kornberg, T., and Lawrence, P. A. 1983. The phenotype of *engrailed* mutations in the antenna of *Drosophila*. *Dev. Biol.* 99: 27–33.

Morata, G., and Lawrence, P. A. 1979. Development of the eye-antenna imaginal disc of *Drosophila*. *Dev. Biol.* 70: 355–371.

Palsson, A., and Gibson, G. 2000. Quantitative developmental genetic analysis reveals that the ancestral dipteran wing vein prepattern is conserved in *Drosophila melanogaster*. *Dev. Genes. Evol*. 210: 617–622.

Patel, N., Martin-Blanco, E., Coleman, K., et al. 1989. Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* 58: 955–968.

Quiring, R., Walldorf, U., Kloter, U., and Gehring, W. J. 1994. Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans. *Science* 265: 742–743.

Salzberg, A., Prokopenko, Y., He, P., et al. 1997. P element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: mutations affecting embryonic PNS development. *Genetics* 147: 1723–1741.

Scanga, S., Manoukian, A., and Larsen, E. 1995. Time- and concentration-dependent response of the *Drosophila* antenna imaginal disc to *Antennapedia*. *Dev. Biol.* 169: 673–682.

Schneuwly, S., Klemenz, R., and Gehring, W. J. 1987. Redesigning the body plan of Drosophila by ectopic expression of the homoeotic gene Antennapedia. *Nature* 325: 816–818.

Tabata, T. Schwatrz, C., Gustavson, E., Ali, Z., and Kornberg, T. 1995. Creating a Drosophila wing de novo, the compartment border hypothesis. *Development* 121: 3359–3369.

Waddington, C. H. 1961. Genetic assimilation. In *Advances in Genetics.* Vol. 10: 257–329.

Wagner, G. P., and Laublicher, M. D. 2000. Character identification in evolutionary biology: the role of the organism. *Theoretical Biolsciences* 119: 20–40.

White, R. A. H. 1998. Immunolabeling of Drosophila. In D. B. Roberts (ed). *Drosophila*. Oxford University Press, Oxford, pp. 215–240.

Williams, T. A., and Muller, G. B. 1996. Limb development in a primitive crustacean, Triops longocaudatis: subdivision of the early limb bud gives rise to multibranched limbs. *Dev. Genes Evol.* 206: 161–168.

Wimmer, E. A., Carleton, A., Harjes, P., Turner, T., and Desplan, C. 2000. Bicoid-independent formation of thoracic segments in Drosophila. *Science* 287: 2476–2479.

Zwick, P. 2000. Phylogenetic system and zoogeography of Plecoptera. *Annual Review of Entomology* 45: 709–746.