

# Hypothesis Testing in Evolutionary Developmental Biology: A Case Study from Insect Wings

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## Abstract

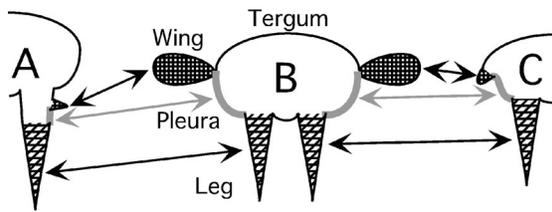
Developmental data have the potential to give novel insights into morphological evolution. Because developmental data are time-consuming to obtain, support for hypotheses often rests on data from only a few distantly related species. Similarities between these distantly related species are parsimoniously inferred to represent ancestral aspects of development. However, with limited taxon sampling, ancestral similarities in developmental patterning can be difficult to distinguish from similarities that result from convergent co-option of developmental networks, which appears to be common in developmental evolution. Using a case study from insect wings, we discuss how these competing explanations for similarity can be evaluated. Two kinds of developmental data have recently been used to support the hypothesis that insect wings evolved by modification of limb branches that were present in ancestral arthropods. This support rests on the assumption that aspects of wing development in *Drosophila*, including similarities to crustacean epipod patterning, are ancestral for winged insects. Testing this assumption requires comparisons of wing development in *Drosophila* and other winged insects. Here we review data that bear on this assumption, including new data on the functions of *wingless* and *decapentaplegic* during appendage allocation in the red flour beetle *Tribolium castaneum*.

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Recent work in evolutionary developmental biology has led to both new hypotheses about how organisms have evolved and the resurrection of old hypotheses. An example of a new hypothesis is that the insect labrum is derived from the endites of the intercalary segment (Haas et al. 2001). Resurrected hypotheses include the hypothesis that vertebrates are upside-down arthropods (originally proposed by St. Hilaire in the 1820s and resurrected by Arendt and Nübler-Jung 1994), the hypothesis that complete metamorphosis in insects evolved by repetition of an embryonic instar (originally proposed by Berlese in the 1910s and resurrected by Truman and Riddiford 1999), and the hypothesis that insect wings evolved from leg branches rather than as de novo outgrowths of the body wall (argued for by Kukulová-Peck 1978 with molecular developmental data contributed by Averof and Cohen 1997). We will use this last example as a case study to explore hypothesis testing in evolutionary developmental biology.

Because developmental data are typically only available from a limited array of species, the hypotheses of homology implied by evolutionary transformations such as those proposed above are often inferred from very limited taxon

sampling; in some cases the data come from only a few distantly related model species. In keeping with the principle of parsimony, similarities between distantly related organisms are inferred to be ancestral. For instance, similarities in how arthropod and vertebrate appendages are patterned have led to the suggestion that arthropod and vertebrate limbs are homologous (De Robertis and Sasai 1996). In this case, substantial morphological and paleontological evidence argues strongly against this conclusion, leading others to conclude that the patterning similarities resulted from parallel co-option of developmental regulatory networks (e.g., Shubin et al. 1997). These two scenarios lead to different inferences about the nature of the last common ancestor of arthropods and vertebrates, highlighting the role of ancestral state inferences in hypothesis testing in evolutionary developmental biology. If co-option of regulatory networks is common in development, then the evaluation of ancestral state inferences requires dense taxon sampling as well as considerations of nonparsimonious explanations for the data. In this article, we use data on insect wing development and evolution to illustrate how hypotheses of homology and co-option can be evaluated



**Figure 1.** Competing hypotheses for the evolutionary origin of wings. Diagrammatic cross sections show (A) the ancestor of winged insects hypothesized by the wings-from-legs theory; (B) an extant winged insect; (C) the ancestor of winged insects hypothesized by the paranotal lobe theory. Colors, patterns, and arrows show the proposed structural homologies between the hypothetical ancestors and modern winged insects.

independently. We begin by reviewing the major hypotheses concerning the evolutionary origin of wings, and then identify and evaluate the assumptions that are made when developmental data are used to support the hypothesis that the wing evolved from a branch of the ancestral arthropod leg. After reviewing evidence in favor of this hypothesis of homology, we consider the evidence needed to test hypotheses of co-option, which could explain the similarities in dorsal appendage patterning in different arthropod lineages if wings evolved de novo.

## How Did Insect Wings Evolve?

Insect wings are an evolutionarily significant novelty whose origin is not recorded in the fossil record. Insects with fully developed wings capable of flight appear in the fossil record in the upper Carboniferous (ca. 320 million years ago), by which time they had already diversified into more than 10 orders, at least 3 of which are still extant. Wingless insects are observed in the fossil record as early as the Silurian (ca. 400 million years ago) (Engel and Grimaldi 2004). The intervening fossil record is poor and no fossils showing intermediate stages in the evolution of wings have been identified (Kukalová-Peck 1991). Unresolved questions about wing evolution include from what ancestral structure wings evolve, what the ancestral function of wings was, and whether wings evolved in an aquatic or terrestrial lineage. Biologists have looked to additional data sources, such as development (e.g., Averof and Cohen 1997) and functional studies (e.g., Kingsolver and Koehl 1985; Marden and Kramer 1995), to investigate the evolutionary origin of wings. These investigations have given rise to numerous competing hypotheses. The hypotheses about morphological origin can be divided into two major classes based on whether the wing is proposed to represent an essentially new structure, not homologous with structures in any other arthropod group, or a transformed old structure.

Throughout most of the 20th century, insect biologists believed that wings originated de novo from lateral expansions, or paranotal lobes, of dorsal thoracic segments

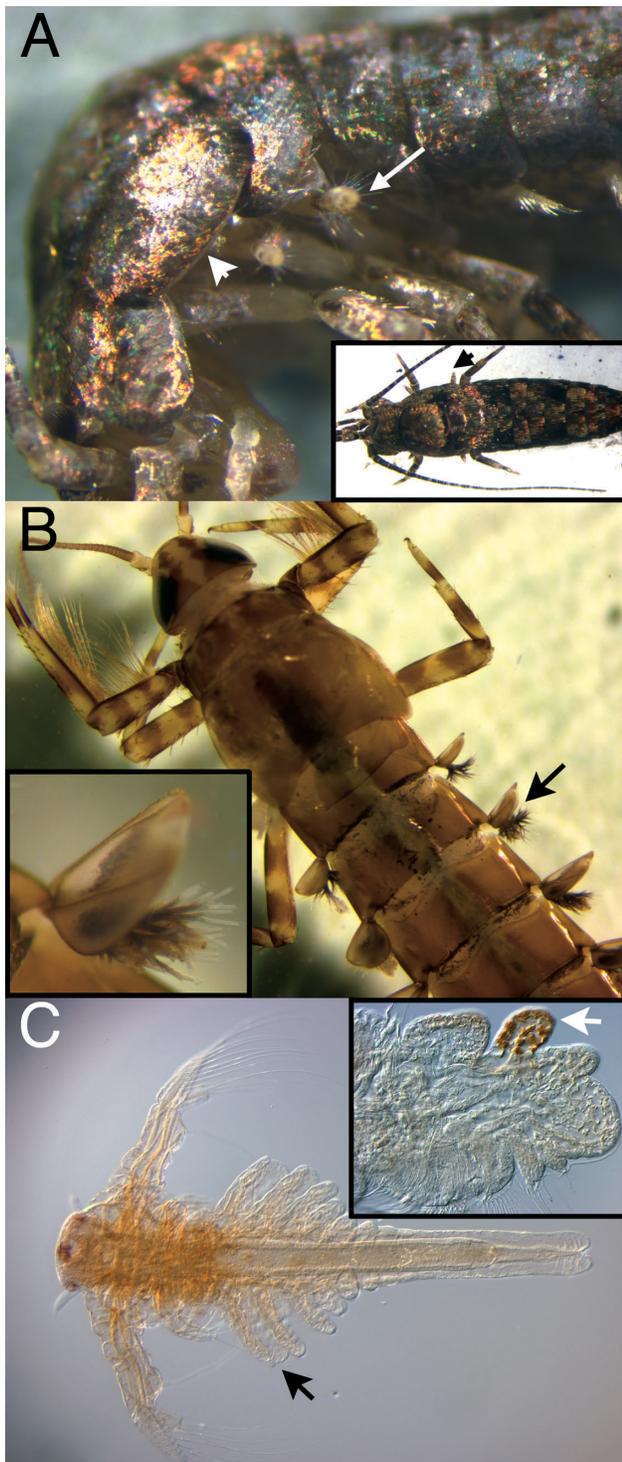
(terga; Figure 1) (Crampton 1916; Snodgrass 1935). The main lines of evidence for this hypothesis are the dorsal location of the wing articulation in adult insects, the presence of tergal expansions in basal and fossil insects (Figure 2), and the fusion of the wing primordia to the terga during development of hemimetabolous species (Brusca and Brusca 1990; Snodgrass 1935). An alternative de novo hypothesis derives the wings from evaginations of the lateral (pleural) rather than dorsal region of the body wall, perhaps as flaps above the spiracles (Bochorova-Messner 1971, as cited in Kukalová-Peck 1978).

More recently, an older theory that the wing is homologous to a proximal branch of the ancestral arthropod leg has been revived and modified. Although almost all extant insects are characterized by unbranched thoracic legs, branched limbs occur in insect mouthparts and in the thoracic legs of one primitively wingless order, the Archaeognatha (Figure 2). They are also found in many living crustaceans (Figure 2) and a diversity of fossil arthropods, suggesting that branched limbs are ancestral for arthropods and thus that ancestral insects may also have had branched thoracic limbs. However, the morphology of ancestral arthropod legs is still debated, as is which portion of the ancestral leg would have given rise to the wing. On the basis of fossil reconstructions, Kukalová-Peck (1983, 1992) argued that primitive arthropod legs were composed of 11 segments, several of which bore lateral extensions (called exites when located dorsally and endites when located ventrally). According to Kukalová-Peck, the most proximal segment of the ancestral leg, the epicoxa, migrated dorsally and evolved into the wing hinge. In this model, the wing is homologous to the epicoxal exite and the pleural region of the body wall is derived from proximal leg segments (Figure 1). Other versions of the wing-from-leg theory derive the wing from the coxal exite of archaeognathans (Wigglesworth 1973, 1976) or from an exite plus endite (Trueman 1990).

Two pieces of developmental data have been used to support the wings-from-legs hypothesis. The first is the observation that in the fruit fly *Drosophila melanogaster*, the leg and wing of each hemisegment (left or right half of a segment) originate as a shared primordium in the ventral region of the blastoderm stage embryo; the wing primordium then separates from the leg primordium and migrates dorsally (Cohen et al. 1993). Similarities in molecular patterning of *Drosophila* wings and dorsal branches of crustacean legs (Averof and Cohen 1997; Nulsen and Nagy 1999) have also been used to conclude that these structures are homologous.

## Assumptions Underlying Use of Developmental Data to Support the Wings-from-Legs Hypothesis

Using developmental data to support the wings-from-legs hypothesis relies on important, but often unstated, assumptions. One assumption is that a shared leg + wing primordium is ancestral for winged insects. If the common



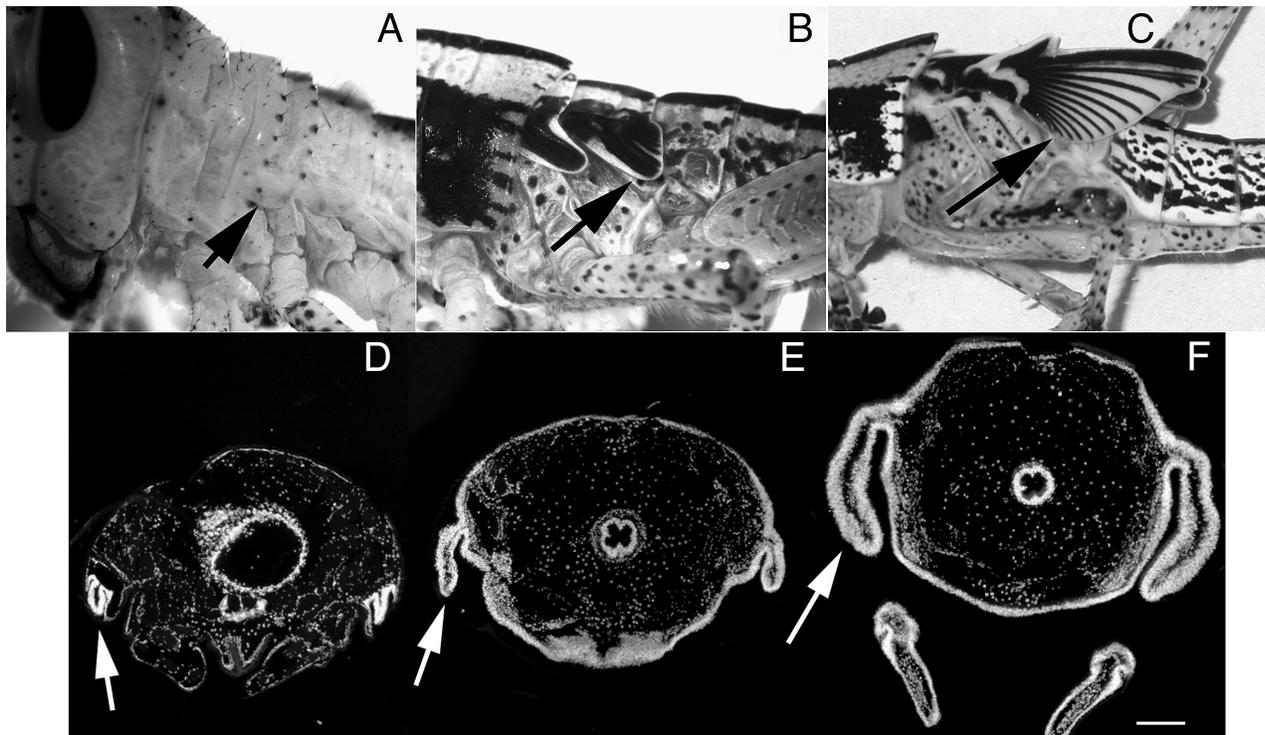
**Figure 2.** Structures hypothesized to be wing homologues. **(A)** Lateral view of an archaean gnathian, *Trigoniphthalmus alternatus*. Arrowhead shows paranotal lobe on the second thoracic segment; arrow indicates coxal stylus of the third thoracic (T3) leg. Inset shows dorsal view; black arrowhead indicates coxal stylus of T3 leg. The coxal styli are sensory structures. **(B)** Dorsal view of a mayfly (*Isonychia* sp.). Arrow indicates abdominal gill; mayfly abdominal gills have been

primordium evolved after wings did, then it cannot be used to support the wings-from-legs hypothesis. Thus the extent to which data from *Drosophila* are relevant to the debate on wing origins depends in part on how much of ancestral wing development has been retained through the 300+ million years separating *Drosophila* from the common ancestor of all winged insects. The support that a common developmental origin provides for the wings-from-legs hypothesis also rests on an ontogeny recapitulates phylogeny type of argument, since it claims that the developmental origin of wings reveals their evolutionary origin. (This assumption will not be discussed further in this article, which focuses on inferences about ancestral wing development; however, the validity of this type of argument has been questioned on a number of grounds, some of which are reviewed in De Queiroz 1985.)

In the case of the resemblance between crustacean leg branches and *Drosophila* wings, the similarity in gene expression patterns is taken as evidence of homology of wings and leg branches, and hence of the evolution of the wing from a leg branch (Averof and Cohen 1997; Nulsen and Nagy 1999). For the similarity to be indicative of homology, the expression patterns shared by *Drosophila* and crustaceans must have been present in the common ancestor of these taxa; thus the *Drosophila* expression patterns would have to be ancestral for winged insects (and the branchiopod pattern would have to be ancestral for crustaceans if insects and crustaceans are sister taxa). However, similarity of gene expression patterns due to common ancestry is not a sufficient condition for homology of the morphological structures, which also requires that the similar structures evolved by modification of the same ancestral structure (Hall 1994). The alternative explanation for similarity in gene expression is convergent (or parallel) evolution, possibly through co-option of the same developmental regulatory network to pattern novel dorsal appendicular structures (Jockusch and Nagy 1997).

The developmental data used to support the wings-from-legs hypothesis rely on hypotheses of ancestral states for insects as a whole, but are generally based on data from a single species, the fruit fly *D. melanogaster*. The best way to infer whether a character is ancestral for a clade is to examine that character in broadly sampled representatives of the clade. In order to assess support for hypotheses about ancestral states of wing development, we review data on the embryology and molecular development of wings in *Drosophila* and investigate the extent to which wing development is similar in other insect lineages. First, we

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proposed to be wing homologues in both wing-from-leg and pleural origin theories (Kukalová-Peck 1978, 1983). Inset shows branched structure, with anterior plate-like branch and posterior ramified branch of gill. **(C)** Ventral view of *Artemia franciscana*. Arrow indicates tip of first trunk limb. Inset shows posterior view of a thoracic limb; arrow indicates dorsal epipod showing expression of *nubbin*.



**Figure 3.** (A–C) Wing development in a typical hemimetabolous species, *Schistocerca americana*. Arrows point to the developing wings. (A) First instar. (B) Third instar. (C) Fifth instar. (D,E) Wing development in a typical holometabolous species, *Tribolium castaneum*. Cross sections through the mid last larval instar (D) and the late last larval instar (E). Sections are DAPI stained to show distribution of nuclei. Arrows show the developing wings.

evaluate the embryological evidence for a shared leg + wing primordium outside of *Drosophila* and conclude that there is no evidence of one in insects other than flies. Second, we evaluate the degree of conservation of the molecular regulation of appendage allocation. In addition to gene expression data from insects with a variety of developmental modes, we present results from RNA interference in the red flour beetle (*Tribolium castaneum*), which was used to evaluate the relationship between gene expression and gene function during appendage allocation in a species with a more ancestral mode of appendage development. Finally, we compare wing patterning in *Drosophila* and other insects to crustacean epipod and insect body wall patterning in order to assess the relative support for the alternative hypotheses of homology and parallel co-option as explanations for similarities between gene expression patterns in dorsal appendages of different arthropod groups.

### Wing Development in *Drosophila* is Highly Derived

*Drosophila melanogaster* is a holometabolous insect, that is, formation of the adult involves a dramatic metamorphosis and a resting pupal stage. In *Drosophila*, virtually the entire adult ectoderm is formed from imaginal discs. The adult appendage primordia are molecularly distinct from the

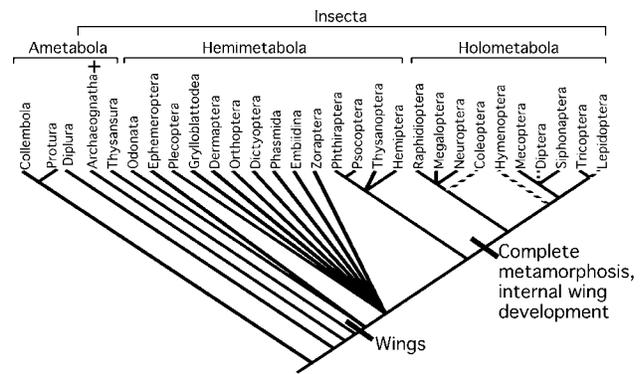
surrounding larval tissue in the embryonic blastoderm (Cohen et al. 1993) and become morphologically distinct when they invaginate from the embryonic ectoderm late in embryogenesis to form imaginal discs, which are flattened epithelial tissues (Cohen 1993). The discs proliferate internally during larval life, and growth and patterning of the disc tissue are integrated (Lecuit and Cohen 1997). At metamorphosis, the discs evert through a process of cell rearrangement to form the adult appendages and adjacent body wall.

Development of *Drosophila* differs from that of other insects in a variety of ways, and several lines of evidence suggest that the *Drosophila* conditions are derived within the lineage leading to higher flies, not characteristic of the ancestral state within winged insects. Numerous extant lineages of winged insects have a hemimetabolous life cycle (i.e., one characterized by incomplete metamorphosis), in which legs develop embryonically and wings grow out directly from the dorsal thorax during juvenile life. The legs attain their basic adult structure prior to hatching and do not undergo a substantial metamorphosis as in *Drosophila* (Snodgrass 1935). In hemimetabolous species, wing development is a gradual, external process in which the wing primordia evaginate from the ectoderm during an early instar and enlarge at each subsequent molt (Figure 3). Representative hemimetabolous species include mayflies (Ephemeroptera), grasshoppers (Orthoptera), and true bugs (Hemiptera). The

widespread taxonomic distribution of hemimetabolous development suggests that hemimetaboly is ancestral for winged insects (Figure 4). Kukulová-Peck (1978) has argued that hemimetaboly evolved in parallel in many lineages and that the common ancestor of winged insects was ametabolous (lacking metamorphosis); in this scenario, ancestral wing development is also gradual and external.

Holometaboly is believed to have evolved only once, in the common ancestor of a large clade of insects that includes flies, moths, beetles, and bees (Figure 4). In most holometabolous species, legs develop as direct outgrowths of the ectoderm during embryogenesis, just as they do in hemimetabolous species. Thus the evolution of holometaboly does not appear to have involved major changes in early leg development (although leg segmentation is not complete in holometabolous larvae, and legs attain their final form at metamorphosis) (Snodgrass 1935). Holometabolous species undergo internal wing development and the wing primordia are not evident externally until the pupal stage, at which point they are fully formed. In some holometabolous groups, such as *Drosophila*, wings develop from imaginal discs. Although holometaboly is commonly equated with the presence of wing imaginal discs (e.g., Chapman 1999), in many holometabolous species, wing development is initiated by evagination of the wing primordium in the last larval instar (Svacha 1992; Truman and Riddiford 1999). The wing tissue proliferates rapidly in a short window of time immediately prior to pupation, and the proliferation results in an elongated, evaginated wing primordium (Figure 3) (Quennedey and Quennedey 1999). In these holometabolous groups, the wings develop as direct outgrowths of the ectoderm, just as they do in hemimetabolous taxa. Following Svacha (1992), we do not consider these late-developing wing primordia imaginal discs since developmental criteria that would group these wing primordia with imaginal discs would also include the wing primordia of hemimetabolous taxa. The biggest difference between wing development in hemimetabolous lineages and holometabolous groups in which wings develop directly is the timing of wing development: it is spread out over multiple juvenile instars in hemimetabolous groups, but compressed into the end of the final instar in many holometabolous lineages. Late development of wings is believed to be ancestral for holometabolous insects, with early invaginating imaginal discs having evolved multiple times independently, including in the lineage leading to higher flies (Figure 4) (Svacha 1992; Truman and Riddiford 1999).

There are several additional ways in which the ancestral form of holometabolous wing development may have more in common morphologically with hemimetabolous wing development than with imaginal disc development. In hemimetabolous and ancestral holometabolous development, the cells that form the adult wing also participate in juvenile development, for example, by secreting cuticle (Svacha 1992). The spatial arrangement of cells is also more similar in hemimetabolous and ancestral holometabolous wings than either is to the cell arrangement in *Drosophila* imaginal discs. At metamorphosis in *Drosophila*, intercalation of cells and eversion of the disc converts the flattened



**Figure 4.** Relationships of hexapod orders based on Kristensen (1991). Evolutionary events affecting wing development are indicated. Hatched lines indicate the orders within which early invaginating imaginal discs evolved. Members of the order Archaeognatha, marked with +, have dorsal leg branches.

imaginal disc to a tubular appendage primordium. Prior to disc eversion, dorsal and ventral cells that end up apposed to each other are separated along the diameter of the disc and the center of the disc forms the distal tip of the appendages. In contrast, in direct-developing appendages, the arrangement of cells in the appendage primordium more closely resembles their tubular arrangement in the adult appendage, with dorsal and ventral regions apposed to each other throughout development.

By inferring evolutionary changes in a phylogenetic framework, it is clear that the following features of *Drosophila* appendage development evolved after the evolutionary origin of wings (Figure 4): (1) internal wing development, which originated in the common ancestor of the Holometabola; (2) the evolution of leg imaginal discs, which is coincident with the loss of larval legs in the common ancestor of flies (Peterson 1960); and (3) the evolution of wing imaginal discs within flies (Truman and Riddiford 1999). The timing of wing and leg development was decoupled throughout much of insect evolution, raising the possibility that the early association between them observed in *Drosophila* evolved secondarily, in conjunction with the evolution of extreme separation between larval and adult fates. Given the substantial developmental changes that distinguish wing development in *Drosophila* from wing development in other pterygotes, it is essential that the assumption that any feature of *Drosophila* wing development is ancestral for winged insects be further tested.

## Do Shared Appendage Primordia Exist in Insects Other than Flies?

The assumption that a shared appendage primordium is ancestral for winged insects is implicit in the argument that the existence of such a primordium in *Drosophila* supports a leg branch origin for the insect wing. The existence of

a shared primordium in *Drosophila* is supported by multiple lines of evidence. A close physical association between the leg and wing primordia was first shown using fate maps constructed from gynandromorphs and clonal analysis (Lawrence and Morata 1977; Wieschaus and Gehring 1976) and has been confirmed more recently using molecular markers. In the shared primordium, both the presumptive wing and leg cells express the homeobox transcription factor *Distal-less* (*Dll*). Normally *Dll* is repressed in the most dorsal of these cells, which segregate from the leg primordium, migrate dorsally, and invaginate to form the wing disc (Cohen et al. 1993; Kubota et al. 2000). The expression of *Lac-Z* under a *Dll* promoter persists longer than the expression of native *Dll*, allowing the dorsally migrating cells to be followed until expression of a wing-specific marker is initiated (Cohen et al. 1993). In addition, expression of the earliest known wing-specific markers, *snail* (*sna*) and *vestigial*, is initiated in the wing primordium just after it separates from the leg primordium, and the dorsal migration of *sna* expression has been observed with in situ hybridization (Fuse et al. 1996).

While a shared primordium has been convincingly shown in *Drosophila*, the assumption that a shared primordium is ancestral has not been closely examined. To date, a close embryological association between wing and leg primordia has only been demonstrated in other species of flies in which both wings and legs develop from imaginal discs. In *Dacus tryoni* (Tephritidae) and *Tipula saginata* (Tipulidae), a physical connection between the leg and wing imaginal discs is observed late in development (Anderson 1963; Birket-Smith 1984), suggesting that they have a common origin. A contrasting pattern is observed in two species of hymenoptera with early invaginating leg and wing imaginal discs, the honeybee (*Apis mellifera*) and a parasitic wasp, *Habrobracon juglandis*; in these, fate maps based on gynandromorphs indicate that the wing and leg primordia are separate in the blastoderm stage (Milne 1976; Petters 1977). In a diversity of species without imaginal discs, dorsal migration of epidermal thickenings that give rise to the wing primordia has been observed (Kukalová-Peck 1978; Tower 1903). However, these observations do not provide support for a shared leg + wing primordium because the epidermal thickenings are always first observed dorsal to the spiracle, while legs develop ventral to the spiracle (Kukalová-Peck 1978). Thus all available data suggest that in insects other than flies, the leg and wing primordia are separated from the earliest developmental stages at which they have been identified, so the assumption that a shared primordium is ancestral for winged insects is untenable at present.

It is possible that a shared leg + wing primordium occurs in insects other than flies but has not yet been described. In *Drosophila*, the initial allocation involves a small number of morphologically undifferentiated cells. Even at invagination, the wing disc primordium contains only about 50 cells (Williams et al. 1993). Observations of wing development in other species have been almost exclusively based on morphology (e.g., Quenndey and Quenndey 1999; Tower 1903), which would not detect the shared appendage

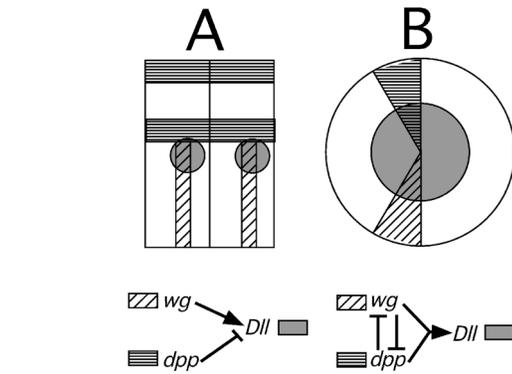
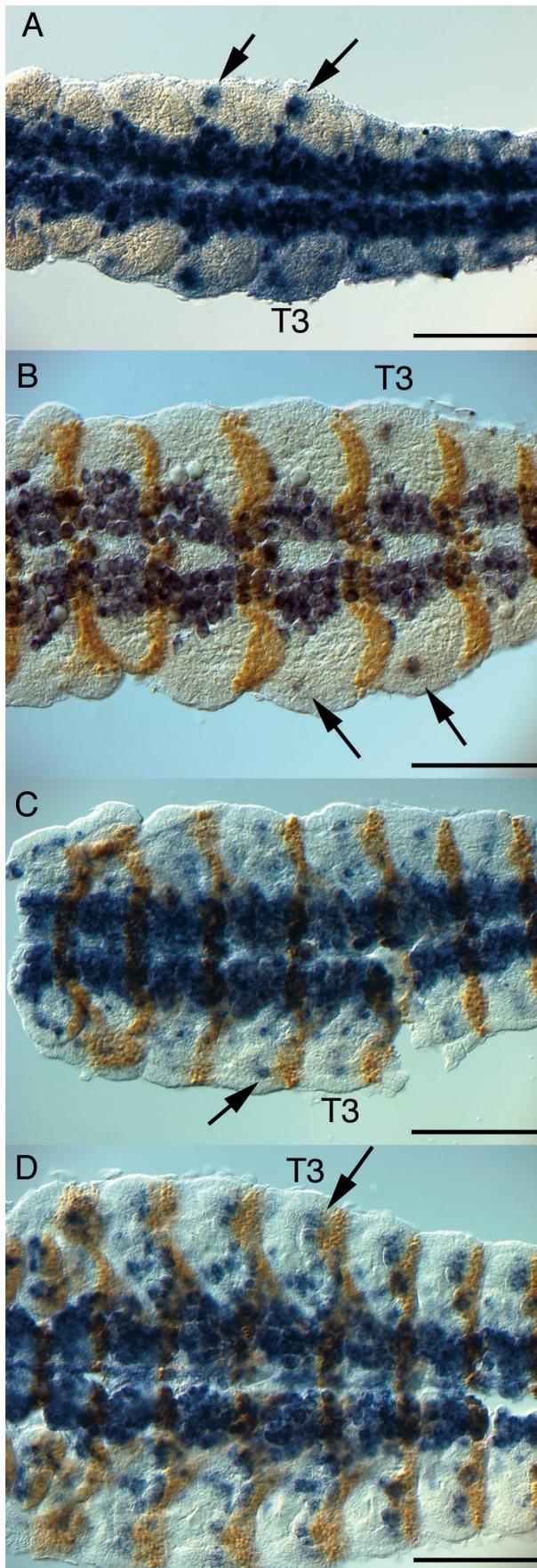
primordia in *Drosophila*. Lineage tracing techniques and molecular markers both offer the potential to identify wing primordia before they are detectable morphologically, and should be used in an array of insects with different developmental modes in order to determine whether legs and wings originate from a common primordium in other lineages.

In a search for candidate wing primordia prior to wing evagination, we have used in situ hybridization to characterize the expression of one of the earliest wing disc markers of flies, *sna*, in *T. castaneum*, a beetle in which wings develop late in the last larval instar and legs develop during embryogenesis. In midembryogenesis, we observe patches of *sna* in the second and third thoracic segments, the wing-bearing segments (Figure 5). By double labeling with an Engrailed (*En*) antibody (4D9) (Patel et al. 1989), which characterizes the posterior region of each segment, we have confirmed that these patches lie at the boundary between *En*-expressing and nonexpressing cells, the anteroposterior position at which wings are expected to develop (Tabata et al. 1995; Zecca et al. 1995); they are also dorsal to the spiracles, the dorsoventral position at which wings are expected to develop (Kukalová-Peck 1978). Similar *sna*-expressing patches are present in the first thoracic segment and in abdominal segments (Figure 5), but this does not rule out a role for these patches in wing development, as ancestral winged insects may have had wings on every segment (Kukalová-Peck 1978). These *sna*-expressing domains raise two questions: where the cells originate and whether these cells give rise to wings.

Examination of *sna* expression earlier in embryogenesis suggests that the *sna*-expressing patches originate further anteriorly in the segment and then migrate posteriorly (Figure 5). Earlier in embryogenesis, the *sna*-expressing cells lie far anterior to the *En* stripe and dorsal to the legs. Gradually the *sna* expression domain moves posteriorly until it overlaps the *En* stripe. Since *sna* expression is characteristic of migrating cells in both flies and vertebrates (Hemavathy et al. 2000), it is likely that the movement of the *sna*-expressing domain results from an anterior to posterior migration of a cluster of cells in each hemisegment rather than from a wave of gene expression through a stationary field of cells. There is, however, no suggestion of a ventral to dorsal migration, as would be expected if wings originated from a shared appendage primordium. These data thus raise the possibility that wing cells are distinct early in embryogenesis, as in *Drosophila*, but originate separately from the legs. Lineage tracing is needed to further investigate the origin and fate of this cell population.

## How Conserved is the Molecular Regulation of Appendage Allocation?

In addition to embryological data, comparative molecular data on appendage allocation are expected to give insight into the degree of conservation of early appendage development. The developmental networks responsible for the



**Figure 6.** Regulation of *Distal-less* during appendage development in *D. melanogaster*. Expression of and interactions among *wg*, *dpp*, and *Dll* are shown during (A) embryogenesis (schematic shows lateral view of two thoracic segments) and (B) imaginal disc development.

allocation of cells to the shared leg + wing primordium of *Drosophila* are relatively well characterized. The shared appendage primordia of *Drosophila* are allocated at the intersection of two secreted signaling proteins, Wingless (Wg) and Decapentaplegic (Dpp). During appendage allocation, *wg* is expressed in a dorsoventral stripe across the ventral portion of each segment (Cohen et al. 1993) and *dpp* is expressed in two longitudinal stripes, a lateral one that intersects the dorsal edge of *wg* expression and a dorsal one along the dorsal-most edge of the developing embryo (Figure 6) (Goto and Hayashi 1997). An essential role for *wg* in imaginal disc allocation was shown using temperature-sensitive *wg* mutants. When these were shifted to a restrictive temperature prior to appendage allocation, flies showed no evidence of either leg or wing disc primordia, as assayed by the expression of a disc-specific molecular marker (Cohen et al. 1993). *dpp* appears to be specifically required for allocation of cells to the wing rather than the leg portion of the shared primordium, as embryos mutant for the *dpp* signal-transducing gene *Thick-veins (Tkv)* lack wing discs but not leg discs (Kubota et al. 2003). In addition, increased *dpp* signaling leads to an increased number of wing cells (Kubota et al. 2003). The effects of *dpp* on *Dll* expression (see below) suggest that it may also be necessary to prevent more dorsal regions of the embryo from assuming an appendage fate.

*Dll* is one of the earliest markers of appendage fate and its expression in the *Drosophila* embryo is regulated by *wg* and

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**Figure 5.** Expression of *snail* (purple) and Engrailed (brown) in developing *Tribolium* embryos. T3, third thoracic segment. Scale bars indicate 100 μm. (A) Prominent *snail* patches (arrows) in T2 and T3 dorsal to the legs; note the similar, but fainter patches in more anterior and posterior segments. (B) At 30% embryogenesis, thoracic *snail* patches are near the anterior border of the segment. (C) 35% embryogenesis. (D) By 45% embryogenesis, the *snail* patches overlap the En-positive cells.

*dpp*. *Dll* is initially expressed throughout the shared appendage primordium and is subsequently down-regulated in the cells that contribute to the wing primordium (Cohen et al. 1993). Loss of *wg* leads to loss of *Dll* expression (Cohen et al. 1993), while loss of *dpp* expression leads to dorsal expansion of the *Dll* domain during embryogenesis (Goto and Hayashi 1997). Despite the correlation between *Dll* expression and appendage fate, *Dll* is not required for formation of the discs, as demonstrated by the ability to recover discs from fragments of *Dll*-null mutants (which are lethal) that have been cultured in wild-type larvae (Cohen et al. 1993). Although not required for the formation of imaginal discs, *Dll* is essential for later development of distal limb structures (Cohen and Jürgens 1989) (Figure 6B) and provides a molecular marker for early appendage development in *Drosophila*.

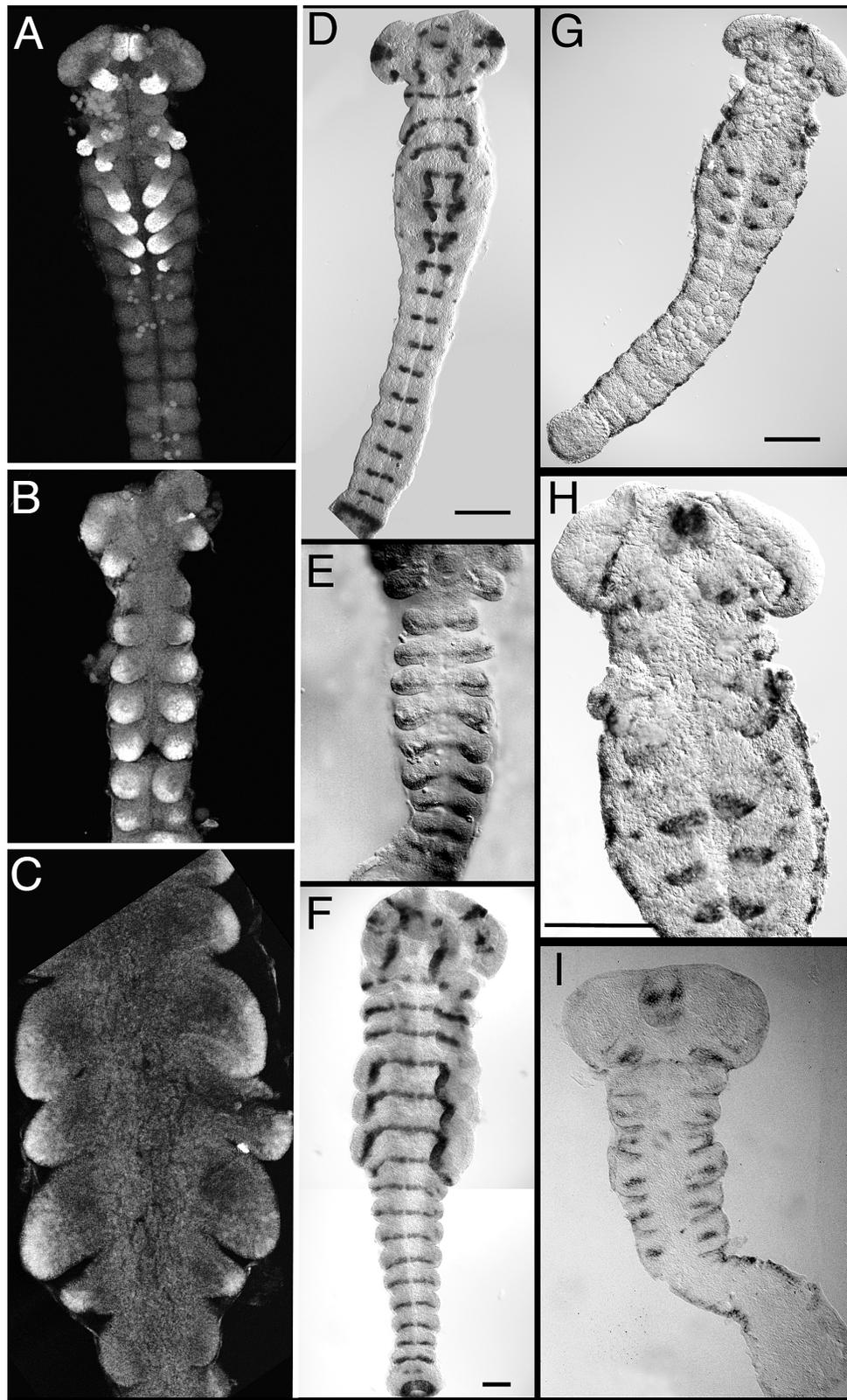
Because of the availability of an antibody to a conserved epitope of *Dll*, relatively extensive comparative expression data are available for this portion of the appendage development network. *Dll* expression has been examined in insects with different leg development modes, and it is conserved in representative ametabolous, hemimetabolous, and holometabolous species (Figure 7) (Beermann et al. 2001; Jockusch et al. 2000; Panganiban et al. 1994; Rogers et al. 2002) as well as in other arthropods (e.g., Abzhanov and Kaufman 2000; Panganiban et al. 1995; Popadic et al. 1998; Prpic and Tautz 2003; Schoppmeier and Damen 2001; Williams et al. 2002). As during *Drosophila* imaginal disc allocation, *Dll* is initially expressed in a single domain in each thoracic hemisegment before the appendage is morphologically distinct. Whether down-regulation occurs in a subset of these cells is unknown, but following limb outgrowth, *Dll* expression is restricted to the distal limb region in all insect species examined. A conserved role for *Dll* in leg development is further supported by mutant analyses in *Tribolium* (Beerman et al. 2001) and by RNA interference in *Tribolium* (Bucher et al. 2002) and a spider (Schoppmeier and Damen 2001).

Expression of *wg* is also highly conserved across the insect species examined, and in some noninsect arthropods. In both *Tribolium* and *Schistocerca americana*, a grasshopper characterized by hemimetabolous development, *wg* is expressed in segmentally reiterated stripes (Figure 7) (Dearden and Akam 2001; Friedrich and Benzer 2000; Jockusch et al. 2000, 2004; Nagy and Carroll 1994). This expression pattern predates the evolution of wings, as it is also found in *Thermobia*, a primitively wingless thysanuran with ametabolous development (Figure 7), and the branchiopod crustacean *Triops longicaudatus* (Nulsen and Nagy 1999). In the three insect species, as in *Drosophila*, the *wg* stripes initially extend from one dorsal edge to the other, with all but the dorsal-most cells expressing *wg*. During limb outgrowth, *wg* expression is absent dorsally, so that the *wg* stripes extend from the tip of one limb across the ventral midline to the tip of the other limb. The similar expression patterns in many arthropods prior to and during appendage outgrowth suggest that *wg* may have conserved functions during development, including in appendage allocation and *Dll* regulation.

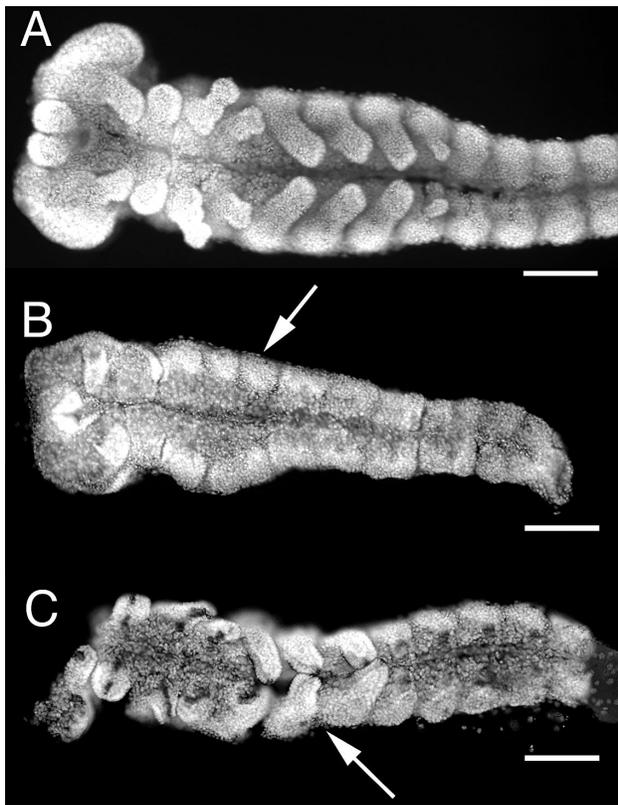
To evaluate this hypothesis of functional conservation, we are using parental RNA interference (RNAi) (Bucher et al. 2002) to create loss of function phenotypes in the flour beetle *T. castaneum*. Injection of gene-specific double-stranded RNA can down-regulate a gene of interest in the embryo (Fire et al. 1998). Analysis of the resulting morphological defects provides insight into the roles of these genes in appendage allocation. The most severely affected *wg* RNAi embryos lack all traces of their thoracic limbs (Figure 8), indicating that *wg* is needed for the formation or outgrowth of legs. We can exclude an alternative explanation, that the defects result solely from the effects of *wg* on *Dll* regulation during later limb patterning, when *wg* is also an activator of *Dll* in *Drosophila* (Lecuit and Cohen 1997) (Figure 6B). A null mutant of *Tribolium Dll* loses its distal limbs, but the limb bases still develop (Beerman et al. 2001), which is less severe than the *wg* RNAi phenotype. Thus data from *Drosophila* and *Tribolium* suggest that *wg* plays similar roles in the initiation of appendage development in a species with imaginal discs and a species in which legs develop directly.

Expression patterns of *dpp* are more dynamic within species and vary more across species than do the expression patterns of *wg*. To date, *dpp* expression in insects has only been described in *Drosophila* (e.g., Ferguson and Anderson 1992; Goto and Hayashi 1997), *Tribolium* (Jockusch et al. 2004; Sanchez-Salazar et al. 1996), and three orthopterans (Dearden and Akam 2001; Jockusch et al. 2000; Niwa et al. 2000). A dorsal longitudinal stripe occurs in both *Tribolium* and *Schistocerca* after formation of the embryonic rudiment and persists until late in embryogenesis (Figure 7). Lateral patches of *dpp* appear in both species, and extensions of these patches result in an expression domain resembling an irregular longitudinal stripe near the base of the limb, as during appendage allocation in *Drosophila*; however, these changes occur substantially after the initiation of limb outgrowth (Figure 7) and do not result in an intersection between *wg* and *dpp*. Thus, in *Schistocerca* and *Tribolium*, *dpp* is not present in the region where it is used to repress *Dll* dorsally during appendage allocation in *Drosophila*. The observation of divergent *dpp* expression during early embryogenesis in grasshoppers led Jockusch et al. (2000) to propose that the allocation phase of appendage development is not conserved across taxa. This hypothesis is strengthened by the observation that the *Tribolium dpp* expression pattern is also quite divergent from the *Drosophila* one, but requires functional testing.

Our data from RNA interference suggest that *dpp* is not required for leg outgrowth in *Tribolium*. The legs in embryos in which *dpp* has been down-regulated using RNAi appear normal (Figure 8). This result is surprising, given that loss of *dpp* function during appendage allocation in *Drosophila* leads to dorsal expansion of the *Dll* domain (Goto and Hayashi 1997). It is also surprising because later in *Drosophila* leg disc patterning, *dpp* acts cooperatively with *wg* to activate *Dll*, and loss of *dpp* during this later stage leads to the loss of distal limb regions (Figure 6B). The *dpp* RNAi embryos lacked the dorsal edge of the body, where *dpp* is also expressed,



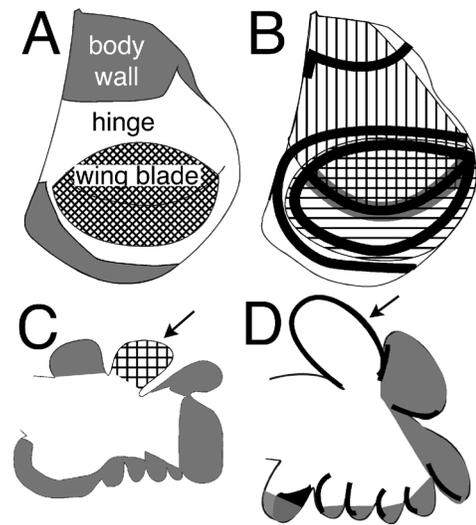
**Figure 7.** Expression of *Dll* (A–C), *wg* (D–F), and *dpp* (G–I) in *T. castaneum* (A,D,G,H), *Thermobia domestica* (B,E), and *S. americana* (C,F,I). Scale bars indicate 100 $\mu$ m.



**Figure 8.** Effects of RNAi on *Tribolium* limb development. Scale bars indicate 100  $\mu\text{m}$ . Embryos are DAPI stained to show the distribution of nuclei. **(A)** Uninjected control embryo with a normal phenotype. **(B)** Embryo injected with double-stranded *wg* RNA, showing absence of limbs and a reduced number of segmental boundaries. Arrow indicates thoracic region. **(C)** Embryo injected with double-stranded *dpp* RNA. Limbs appear normal, but embryo has a shortened dorsoventral axis (arrow) compared to control embryos.

indicating that RNAi was down-regulating gene function in these embryos. Therefore these RNAi data suggest that *dpp* is either not used or functions redundantly in limb development in *Tribolium*, in contrast to its essential role in *Drosophila* limb development.

Because legs develop embryonically and wings develop late in larval life in *Tribolium*, we have focused on assessing the role of *Drosophila* appendage allocation genes in leg development rather than wing development. The comparative functional data from *Tribolium* suggest that the roles of *dpp* in both allocation and later limb patterning have diverged; however, the function of *wg* during early appendage allocation, as assayed by leg development, may be conserved between *Drosophila* and *Tribolium*. While the differences in *dpp* function and expression are intriguing, data from additional taxa are needed to infer the ancestral function of *dpp* and the direction of evolutionary change. Our data suggest that some significant aspects of leg allocation differ between two insect holometabolous species with different modes of leg de-



**Figure 9.** Comparisons of gene expression in insect wings and the multibranching of crustacean limbs. **(A)** Fate map of the *Drosophila* wing imaginal disc (based on Bryant 1975), with shading indicating different fates as labeled. Anterior is to the left and dorsal is toward the top. The line in the middle of the wing blade indicates the dorsoventral boundary. In **(B–D)**, shading and patterns are used to indicate the expression domains of the following genes: *apterous* (vertical stripes), *nubbin* (horizontal stripes), *wingless* (black), and *Distal-less* (gray) in a late third instar wing imaginal disc of *D. melanogaster* **(B)**; based on Campbell and Tomlinson 1998; del Álamo Rodríguez et al. 2002; Neumann and Cohen 1998; Williams et al. 1993); a multibranching trunk limb of the brine shrimp *Artemia franciscana* **(C)**; based on Averof and Cohen 1997; Panganiban et al. 1995); and a multibranching trunk limb of the tadpole shrimp *Triops longicaudatus* **(D)**; based on Nulsen and Nagy 1999; Williams et al. 2002).

velopment. These species also differ in mode of wing development, and additional functional data are needed to test whether divergence in leg allocation is correlated with divergence in wing allocation in *Tribolium*.

### Patterning Similarities Between Insect Wings and Crustacean Epipods

The wings-from-legs hypothesis has received support not only from the earliest stages of wing development in *Drosophila*, but also from later patterning similarities between *Drosophila* wings and dorsal branches of crustacean legs. Later wing disc patterning has been well characterized in *Drosophila*, and in this section we limit our discussion to genes for which some comparative data are available that bear on the wings-from-legs hypothesis. In *Drosophila*, the wing disc gives rise not only to the wing, but also to the wing hinge and adjacent body wall. Although the wing disc primordium is already distinct from the surrounding ectoderm during

embryogenesis, the distinction between wing and body wall tissue in the disc does not arise until the early second instar (Wu and Cohen 2002). Genes that are critical for proper wing disc development include *wg* and two transcription factors, *nubbin* (*nub*), which encodes a POU domain protein, and *apterous* (*ap*), which encodes a LIM homeodomain protein. Expression of *Wg* is highly dynamic. Early in the second instar, it is expressed in an anterior ventral region, where it plays a role in the early distinction between wing and body wall (Williams et al. 1993; Wu and Cohen 2002). By late in the second instar, *Wg* is expressed along the dorsoventral margin of the disc, in response to *ap*. The dorsoventral boundary acts as an organizer of wing development, and one function of *Wg* along this boundary is the induction of *Dll* expression, which is involved in patterning the wing margin (Zecca et al. 1996). Unlike in the leg, *Dll* is not required for outgrowth of the wing (Cohen and Jürgens 1989). During the third instar, three additional *Wg* domains appear: two rings of expression in the proximal wing region, and a patch in the dorsal body wall (del Álamo Rodríguez et al. 2002; Whitworth and Russell 2003). Expression of *ap* is induced throughout the dorsal region of the wing disc in the middle of the second instar and maintained throughout development. Although its expression is restricted to the dorsal region, loss of *ap* leads to loss of the entire wing, indicating a global role in wing patterning (Blair et al. 1994; Diaz-Benjumea and Cohen 1993). Expression of *nub* throughout the wing and wing hinge region is also regulated by *wg*, and expression in the hinge region plays a role in proximodistal axis patterning of the developing wing (Ng et al. 1995, 1996).

Before reviewing the similarities with crustacean limb patterning, we ask to what extent *Drosophila* wing patterning networks are conserved in other species of insects. Gene expression patterns during wing development have only been examined in two insect orders in addition to the Diptera, the Lepidoptera and Hymenoptera, and only in representatives of those orders characterized by early invaginating imaginal discs (these are believed to represent convergently evolved imaginal discs) (Svacha 1992; Truman and Riddiford 1999). As in flies, *wg* is expressed along the dorsoventral margin of the wing disc in butterflies (Carroll et al. 1994; Weatherbee et al. 1999) and several species of ants (Abouheif and Wray 2002). Expression of *ap* has been reported from a butterfly, *Precis coenia*, where it is restricted to the dorsal wing region (Carroll et al. 1994). *Dll* is expressed along the dorsoventral margin of the wing in butterflies, as well as in wing eyespots, which are unique to Lepidoptera (Brunetti et al. 2001; Carroll et al. 1994). Expression of *nub* during wing development has not been reported for any other insect species. Thus, to date, comparative data suggest that wing patterning in *Drosophila* is shared with a clade containing part of the Holometabola, but the lack of data from other lineages precludes claims about ancestral states deeper in the insect tree.

Although no definitive homologue of wings has been identified outside of insects, one candidate homologue is a dorsal branch of ancestral arthropod legs (Averof and Cohen 1997; Kukalová-Peck 1978). Dorsal leg branches,

called epipods, occur in the limbs of many extant crustaceans, including the branchiopods, which have flattened, multibranching limbs (Williams 1999). Expression patterns of four genes differentiate the epipod from other branches of crustacean limbs, and in each case, epipod expression patterns have been interpreted as more similar to fly wing expression patterns than to fly leg expression patterns (Figure 9). In the brine shrimp *Artemia franciscana*, which has two epipods, expression of both *Ap* and *Nub* becomes restricted to the distal epipod (Figure 2); *Nub* is initially expressed throughout the limb primordium (Averof and Cohen 1997). *Dll* expression is also absent or weak in the distal epipod of *Artemia*, but is maintained in the proximal one (Panganiban et al. 1995). Similarly, in the fairy shrimp *Thamnocephalus platyurus*, *Dll* expression is down-regulated in the distal epipod late in development, but maintained in the proximal one (Williams et al. 2002). In the tadpole shrimp *T. longicaudatus*, *Dll* is down-regulated in the single epipod (Williams et al. 2002) and *wg* is expressed along the entire margin of the epipod, whereas in most limb branches its expression is restricted to the ventral side, as in insect legs (Nulsen and Nagy 1999). *wg* is not expressed during the development of limbs of another branchiopod, *Mysidium columbiae* (Duman-Scheel et al. 2002).

Thus some epipods resemble insect wings more closely than insect legs in the following ways: *wg* is expressed along the entire margin rather than being restricted to the ventral side, *ap* and *nub* are expressed in large domains, and *Dll* expression is restricted or absent. Significant differences in these expression patterns include *ap* expression throughout the epipod, whereas in wings it is confined to the dorsal compartment, and *wg* expression along the dorsoventral margin of *Drosophila* wings but the anteroposterior margin of branchiopod epipods (Jockusch and Nagy 1997).

### Does Similarity Result from Homology or Co-option?

Two evolutionary scenarios can explain these similarities in gene expression between insect wings and crustacean epipods: independent co-option, and retention of an ancestral patterning network used to pattern an ancestral structure that was transformed into wings in insects and epipods in branchiopod crustaceans. Two conditions must be met for the similarity to result from common ancestry: (1) the structures showing similar gene expression must be homologous and (2) the similarities in gene expression must be ancestral for the clade under consideration. In this case, the differences noted above would result from subsequent divergent evolution in the lineage leading to one or both taxa. In contrast, co-option is hypothesized when homology of the structures is rejected or when the similar expression patterns are not inferred to be ancestral. Co-option appears to occur frequently in evolution, as documented by the overall similarity in sets of developmental regulatory genes across taxa and the many different roles that a single gene or gene network can have in the course of development of

a single species. Co-option of genes is also not random, as evidenced by the parallel independent co-option of *Pax-6*, *Dll*, and *tinman* to pattern eyes, limbs, and hearts, respectively, in both insects and vertebrates. One explanation for this nonrandomness is that genes that are expressed in an appropriate context already (e.g., in primitive photoreceptors in the case of *Pax-6*) are more likely to be co-opted (Panganiban et al. 1997; Wilkins 2002).

Can we discriminate between the hypotheses of ancestral similarity and independent co-option in the case of arthropod appendage evolution? Homology of the underlying structures is under debate, and homology of leg branches even among crustaceans with different limb forms is unresolved (Williams 1999). Homology requires that the similar structures result from modification of the same ancestral structure, which must have been continuously present in both lineages (Hall 1994). One way to test whether this is reasonable in the case of insect wings and branchiopod epipods is to investigate the presence of wings or dorsal leg branches in other descendants of their common ancestor. There are three lineages of primitively wingless hexapods that lack a dorsal appendage branch, and one, the Archaeognatha, with such a branch (Figure 4). This indicates that multiple parallel losses of the hypothesized ancestral structure must be postulated to account for the distribution of wings and putative wing homologues under the wings-from-legs hypothesis.

Since homology of the underlying structures is unclear, it is important to take a two-pronged approach to determining what explains the similar gene expression patterns between wings and epipods: one should test (1) whether the similarities in gene expression are inferred to have been present in the common ancestor of the taxa under consideration, as is necessary (but not sufficient) for an inference of structural homology, and (2) whether patterning in either lineage suggests a reason why co-option of the same genes may have been facilitated, for example, because a shared feature is patterned similarly.

Addressing (1) requires resolution of the crustacean phylogeny, resolution of the relationship between insects and crustaceans, and investigation of gene expression patterns in additional taxa. Addressing (2) requires investigation of patterning in other body regions to determine whether the patterning mechanisms used in wings and epipods are deployed elsewhere in any descendent of the common ancestor of these taxa. The main alternative hypothesis for wing origins is that the wings evolved de novo as body wall extensions (Snodgrass 1935). In branchiopod crustaceans, the multibranching limb primordium covers most of the dorsoventral extent of the embryo and all of the branches appear to originate directly from the body wall (Nulsen and Nagy 1999; Williams and Müller 1996), whereas in taxa with fewer limb branches, the limb primordium occupies only a relatively ventral portion of the body wall (Williams 1999). Ancestral similarities in dorsal body wall patterning could have led to similar patterning of dorsal appendages through independent co-option of body wall patterning networks during insect wing and branchiopod appendage evolution.

Therefore it is important to compare wing and body wall patterning.

Because the dorsal body wall develops from the wing imaginal disc, data on thoracic body wall patterning are available in *Drosophila*. Of interest is that *ap* is expressed throughout the dorsal region of the wing disc, including the portion that gives rise to the dorsal body wall (Blair et al. 1994). Thus *ap* expression is perhaps more a marker of “dorsalness” than it is of “wingedness,” although *Drosophila ap* mutants have relatively normal body walls. Lack of *Dll* expression is also characteristic of body wall patterning in *Drosophila* (Cohen et al. 1993) and other arthropods (e.g., Panganiban et al. 1995; Williams et al. 2002). Its expression along the wing margin (Campbell and Tomlinson 1998) may reflect an ancestral role in sensory structure developments (Mittmann and Scholtz 2001). Retention of this ancestral role in crustaceans could also account for the absence of *Dll* in many epipods, which are often distinguished from other limb branches by the absence of sensory setae (Williams et al. 2002). *wg* is also expressed in discrete patches of the developing body wall in *Drosophila* (Williams et al. 1993), *Tribolium* (Nagy and Carroll 1994; Ober KA and Jockusch EL, unpublished), and *Schistocerca* (Jockusch EL, unpublished). In the latter two taxa, dorsal expression patterns late in embryogenesis appear to correspond to the margin of the developing terga, an expression pattern that resembles expression around the wing margin. Of the genes cited as similar in insect wings and crustacean epipods, only the expression of *nub* is restricted to the future wing region of the wing disc. Thus most features of wing development that are similar to crustacean epipod development also resemble aspects of insect body wall development. Future comparisons of developmental regulatory genes should consider similarities not only between insect wings and crustacean limb branches, but also between insect wings and body wall. It is especially important to include intervening taxa such as thysanurans that primitively lack appendicular extensions of the dorsal body wall, as these may be more likely to retain ancestral body wall patterning mechanisms.

## Conclusion

At the advent of evolutionary developmental biology, biologists were surprised by the degree of conservation in gene expression and function among distantly related taxa such as flies and mice. This apparent conservation led to a sense that extensive taxon sampling was not needed before drawing conclusions about ancestral roles of genes in development. However, as data have become available from more closely related groups of taxa, substantial variation has been noted (e.g., Brunetti et al. 2001; Hughes and Kaufman 2002; Stauber et al. 2002; Stern 1998). Furthermore, conservation of expression does not necessarily indicate conservation of function, even within serially homologous body regions of a single organism (e.g., Gallitano-Mendel and Finkelstein 1997), so functional testing of conserved expression patterns is needed. The existence of variation in

developmental pathways among closely related organisms points to the necessity of greatly increased taxon sampling before making inferences about ancestral states or the direction of evolutionary change among distantly related species.

Model systems, such as *Drosophila*, were chosen for developmental studies because of properties such as rapid development and ease of manipulation (Bolker 1995). The extensive manipulations possible in model systems have revealed many layers of developmental complexity that are inaccessible to study in nonmodel species. However, some of the properties that make *Drosophila* a successful model system are also highly derived, making it a poor representative for insects as a whole. Indeed, any single species is a poor representative of a diverse lineage. The data from model organisms facilitate study of evolutionary diversity by giving us a starting point to identify genes critical for development in other lineages. This opens the door for comparative studies of developmental mechanisms. The full promise of the synthesis of evolutionary and developmental approaches to morphological evolution requires both the depth of data offered by developmental model systems and the breadth of data that can be obtained by sampling organismal diversity.

One of the key contributions that is expected from the synthesis of evolutionary and developmental thinking is insight into the origins of morphological novelties (Gould 1977; Wagner et al. 2000). Inferences about ancestral states play an essential role in testing these hypotheses. As our exploration of the developmental assumptions underlying one of the several competing hypotheses about the evolution of insect wings shows, far more data are needed from phylogenetically important lineages before we can discriminate among these hypotheses. For none of the three aspects of wing development we reviewed were sufficient data available to provide insight into the developmental pathways of ancestral winged insects. We found evidence that a shared leg + wing primordium may be widespread in flies, but there is no evidence that this trait existed deeper in the insect tree. This lack of evidence reflects the lack of data on where, developmentally, wings originate in almost all extant insects. A comparison of the molecular mechanisms underlying appendage allocation revealed that only one of two key genes investigated, *wg*, has a similar role in appendage allocation in *Drosophila* and *Tribolium*, pointing to the need for more comprehensive tests of conservation across taxa. Finally, comparative data suggest that some aspects of later wing patterning may be conserved in the Holometabola, but there are no data from any species that retains an ancestral mode of wing development. Thus claims about wing patterning in ancestral winged insects are based on the assumption that the wing developmental pathways did not change between the most recent common ancestor of winged insects and the most recent common ancestor of Holometabola. Taken together, these three lines of comparison show that the assumptions underlying the use of developmental data to support the wings-from-legs hypothesis are not supported. The evolutionary origin of wings remains an open question,

but one that developmental data hold great promise in addressing as the trend toward comparative studies of development in an ever wider array of taxa continues.

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