# Conservation and Variation in *Hox* Genes: How Insect Models Pioneered the Evo-Devo Field

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## Abstract

Evolutionary developmental biology, or evo-devo, broadly investigates how body plan diversity and morphological novelties have arisen and persisted in nature. The discovery of *Hox* genes in *Drosophila*, and their subsequent identification in most other metazoans, led biologists to try to understand how embryonic genes crucial for proper development have changed to promote the vast morphological variation seen in nature. Insects are ideal model systems for studying this diversity and the mechanisms underlying it because phylogenetic relationships are well established, powerful genetic tools have been developed, and there are many examples of evolutionary specializations that have arisen in nature in different insect lineages, such as the jumping leg of orthopterans and the helmet structures of treehoppers. Here, we briefly introduce the field of evo-devo and *Hox* genes, discuss functional tools available to study early developmental genes in insects, and provide examples in which changes in *Hox* genes have contributed to changes in body plan or morphology.

**Evolutionary developmental biology (evo-devo):** field in biology that addresses processes directing organismal development and how they have changed during evolution to generate diversity

**Genetic toolkit:** genes important for embryonic development, which are highly conserved throughout the animal kingdom

**Homeobox-containing (*Hox*) genes:** a family of clustered homeotic genes sharing a 180-base-pair homeobox that encodes a DNA-binding homeodomain

**Homeotic mutation:** mutations that result in replacement of one body part with an alternate, fully formed body part

## EVO-DEVO AT A GLANCE

Why do some insects use one set of wings for flight and others use two sets? How did the jumping leg of crickets and grasshoppers become disproportionately larger than their other legs? Questions like these are the purview of evolutionary developmental biology, or evo-devo, which studies the basic processes directing organismal development and how they have changed during evolution to promote diversity in body form. Evo-devo encompasses studies of variation in both phenotype and genotype, including embryonic development, morphological novelties, homology, and developmental plasticity, with the larger goal of discovering molecular mechanisms underlying biological diversity (17, 122).

One of the core concepts discovered in evo-devo is that all animals possess a genetic toolkit, or basic collection of genes that control development, which is remarkably conserved throughout the animal kingdom (17). Many toolkit genes encode transcription factors, which function as sequence-specific DNA-binding proteins that activate or repress expression of downstream target genes involved in the formation of specific body structures. A central question in evo-devo raised by this observation is, How can one genetic toolkit produce diverse body plans? An emerging hypothesis is that changes in gene products and/or changes in their expression patterns allow conserved genes to be rewired or co-opted for use in different developmental pathways. Thus, a highly conserved group of transcription factors reorganizes regulatory connections to control the development of diverse organisms (16, 45, 57, 64, 103, 115, 124). Evo-devo studies have contributed to our understanding of organismal development by exploring modes of development in diverse animal systems, and of molecular underpinnings of the evolution of development.

## THE DISCOVERY OF *HOX* GENES IN INSECTS LED TO THE RISE OF EVO-DEVO

Homeobox-containing (*Hox*) genes are fundamental components of the genetic toolkit of metazoans; they are recognized mostly for their role in determining segment identity (17). A century before these genes were cloned, rare mutations were observed in nature, such as insects with legs replacing antennae (5). Bateson (5) coined the term homeosis to describe these aberrations, where "something has been changed into the likeness of something else." Though it would be almost a century before *Hox* genes were isolated and sequenced, and the genetic mechanisms underlying homeotic mutations studied, it was apparent to Bateson that homeosis might play a role in body plan evolution, as he came to the conclusion that "the Study of Variation thus offers a means whereby we may hope to see the process of Evolution" (5).

Elucidation of the genetic basis of insect homeotic mutations began in the mid-1900s through studies of the emerging model organism, the fruit fly, *Drosophila melanogaster* (58, 59). In the 1980s, *Hox* genes were cloned from *Drosophila* (73, 96), and it was a shocking discovery when similar genes were detected in evolutionarily distant species such as beetles, earthworms, and humans (71). This was the first insight biologists had into the genetic toolkit, now so fundamental to evo-devo, and it was the spark that initiated studies in this field.

### *Hox* Gene Clustering, Duplication, and Divergence

Early studies of *Hox* genes are a prime example of curiosity-driven research. E.B. Lewis (reviewed in 58, 59) and others were intrigued by startling homeotic transformations in *Drosophila*, never anticipating that the genes they characterized would be functionally conserved in animals as distant as mouse and human. Using polytene chromosome mapping, these researchers found that mutations causing transformations of posterior body segments (e.g., transformation of haltere to wing) clustered in one region of the third chromosome called the *Bithorax* complex (BX-C) (59). Mutations resulting in homeosis of anterior segments (e.g., transformation of antenna to leg) mapped to another cluster on the third chromosome, the *Antennapedia* complex (ANT-C) (53). In addition to chromosomal clustering, Lewis observed colinearity among homeotic genes: Their linear organization along the chromosome correlated with the region of function along the anterior-posterior axis of the animal (59). *Hox* genes located at the 3′ end of the *Hox* complex (e.g., *labial*) affect body structures in the anterior part of the embryo, while genes at the 5′ end of the complex (e.g., *Abd-B*) affect the posterior region of the animal. After these genes were cloned and expression patterns analyzed, it was quickly realized that their colinear action reflects their anterior-posterior order of expression along the embryonic body axis (2, 8, 59).

The chromosomal clustering and colinearity of *Hox* genes are conserved outside of *Drosophila*, in both invertebrates and vertebrates (**Figure 1**). Most insects have one *Hox* cluster, which is thought to be similar in gene composition to the ancestral *Hox* complex in Urbilateria (22). A single cluster has been maintained outside of vertebrates, as polychaetes (30, 50), onychophorans (37), and sea urchins (15) all have one *Hox* cluster. In vertebrates, there have been *Hox* cluster duplications and paralog-specific gene losses and gains (**Figure 1**). Mammals have four *Hox* clusters (HoxA–D) (95), and teleosts have as many as eight (3, 20). These *Hox* cluster duplications are thought to have facilitated evolutionary radiation and the acquisition of novelties in some lineages (20, 114). The single *Hox* cluster in insects provides an optimal system to examine gene function, as loss- and gain-of-function analyses are not complicated by the presence of multiple *Hox* paralogs and functional redundancy.

After their discovery in *Drosophila*, *Hox* clusters were identified in other insects, including honey bees (117), beetles (105), grasshoppers (27), mosquitoes (25, 85), and moths (126). Whereas *Drosophila Hox* genes are split into two clusters on the same chromosome, *Hox* clusters in these other insects retain the presumed ancestral single cluster (**Figure 1**), with the exception of *labial*, which is located at the opposite end of the chromosome in *Bombyx mori* (126). Thus, while the *Hox* cluster itself appears to be under evolutionary constraint, some cases of split complexes retain function (104). In addition, there have been instances of gene duplication and divergence within insect *Hox* clusters. For example, *B. mori* harbors a tandem duplication of 12 homeobox genes between *pb* and *zen/Hox3* that appears to be unique to this lineage (19), and *Drosophila* and *Tribolium castaneum* carry independent duplications of the *zen* gene (10, 93).

## *Hox* Genes Encode Regulatory Transcription Factors

*Hox* genes share a 180-base-pair homeobox, which encodes a 60-amino-acid homeodomain, so-named because of its discovery in homeotic genes (71, 73, 96). Hox proteins bind DNA via their homeodomains and function as regulatory transcription factors that activate or repress the expression of so-called target or downstream genes. This finding explains the ability of *Hox* genes to regulate entire developmental programs and provides insight into the molecular underpinnings of homeotic transformation (17). Owing to similarities in homeodomains, the DNA-binding sequences recognized by different Hox proteins are similar, yet each Hox protein has a unique and specific role in vivo (the so-called Hox Paradox; 69). One way in which Hox proteins achieve specificity is by interacting with different DNA-binding partners or cofactors, which modulate Hox binding preferences for certain sites in the genome such that each Hox protein regulates a discrete set of target genes (42, 70, 99; see below). Several *Drosophila* and

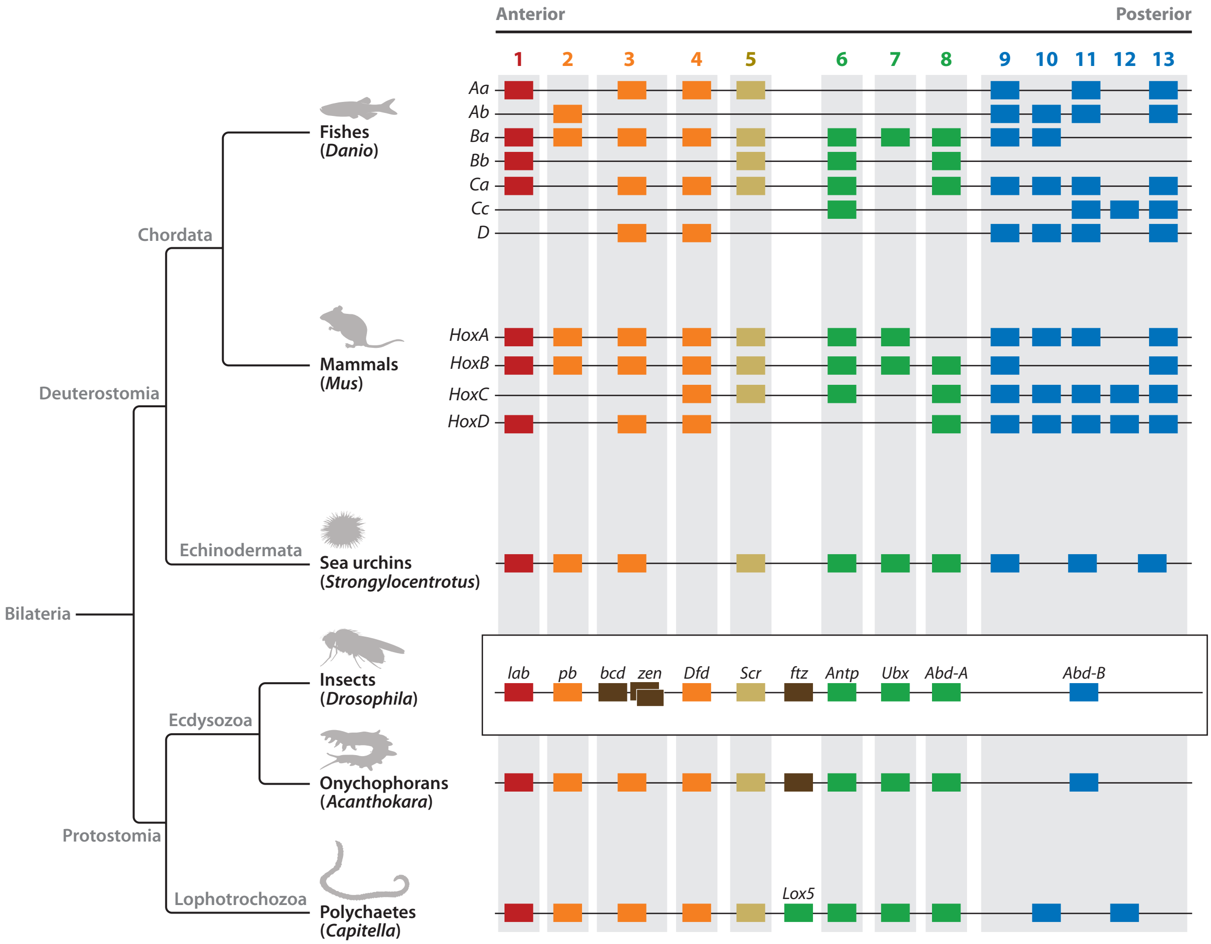


**Figure 1**

*Hox* complexes in bilaterians are highly conserved, despite duplication and divergence of *Hox* paralogs or clusters. Although lophotrochozoans, ecdysozoans, and some deuterostomes have a single *Hox* cluster, there have been lineage-specific duplications of the *Hox* cluster in chordates. Mammals have four *Hox* clusters, with loss of some paralogs within clusters. Fish lineages such as zebrafish have as many as eight clusters. The *Drosophila Hox* complex highlighted in this figure is representative of insects.

mammalian Hox proteins interact with the homeotic cofactor Extradenticle (Exd/Pbx), which increases DNA-binding specificity in vivo (52, 100). Hox functional specificity is also influenced by residues at the amino-terminal end of the homeodomain and by other protein motifs that modulate cofactor interactions and/or transcriptional activity (31, 32, 60, 88, 112, 129, 130; see below).

In sum, *Hox* genes are highly conserved and maintain clustering in metazoans, where they exhibit colinearity of arrangement and expression. Although all *Hox* genes have a DNA-binding homeodomain that recognizes similar binding sequences in the genome, functional specificity is achieved by interaction with cofactors or by the presence of other functional motifs in the protein sequence. While many of the pioneering studies of homeotic genes employed *Drosophila*, species as divergent as flies and humans utilize conserved *Hox* genes for body patterning (72).

# FUNCTIONAL TOOLS AVAILABLE IN INSECTS MAKE THEM GOOD MODELS FOR STUDYING MORPHOLOGICAL DIVERSITY

Insects are an invaluable resource for studying and elucidating the function of many early developmental genes (including *Hox* genes), due largely to the ease of studying these organisms and the numerous functional tools available. Insects are attractive model systems because they generally have short generation times, large numbers of animals can be collected from the field and/or reared in laboratories (often on standardized media or simple food sources), and females lay many eggs, which are often large enough to be seen with the naked eye. Insects are also ideal for evo-devo and comparative studies: They are the most abundant animals on earth, with an estimated 5 million species, representing possibly more than 60% of all known animal species (77). Insects have diverged to occupy virtually every niche on land and with that have acquired a range of elaborate adaptations. A strong history of insect systematics has generated well-established phylogenies that elucidate relationships and divergence times between different insect groups (65). Together, species abundance and diversity, along with the relative ease of experimental manipulation and history of genetic analysis, have generated a rich source of material for large-scale, well-supported comparative analyses in insects.

## *Drosophila* Genetics Leads the Way

The field of *Drosophila* genetics has a long and illustrious history, including numerous landmark discoveries and multiple Nobel Prizes in the last 100 years, beginning with T.H. Morgan, who was awarded the 1933 Nobel Prize in Physiology or Medicine for his discovery of the *white* mutation (35). *Drosophila* has developed into a versatile, accessible, and widely used model system for identification and functional characterization of genes and genomes (14). *Drosophila* genetics rests on a historic collection of mutations that alter gene function: Loss-of-function mutations reveal wild-type gene function in whole animals, and gain-of-function mutations reveal the potential of genes to carry out activities when ectopically expressed or overexpressed. These types of mutations have been generated over many years by a variety of methods, including saturation genetic screens (79), and have been stably maintained in individual labs and stock centers throughout the world (75).

*Drosophila* is similarly unsurpassed in molecular genetics: Even before the genome sequence was available, fly researchers had developed methods to rapidly isolate genes of interest (123) and to analyze the spatiotemporal expression patterns of genes by in situ hybridization and antibody staining (107). A groundbreaking tool for studying gene function in *Drosophila* was the introduction of transgenes by P-element insertion (89). The integration of any DNA sequence into the genome allowed for the rescue of mutants with functional or altered transgenes and for the identification of *cis*-regulatory sequences in vivo. Recent years have seen the development of sophisticated genetic manipulations, including tools for misexpressing genes in specific tissues (UAS/GAL4 system, 9) and for generating tissue- and cell-specific clones (e.g., FLP-FRT, 110; mosaic analysis with a repressible cell marker, 125). This work in *Drosophila* set the stage for the explosion of developmental biology and functional genomics that began in the late-twentieth century (6). The fly genome is essentially saturated with mutations of all types, revealing the function of many of the approximately 13,000 genes in the *Drosophila* genome. Accordingly, *Drosophila* serves as a reference for gene categorization from virtually any species: If a gene is cloned from a nonmodel insect, its function is hypothesized on the basis of the known function of the *Drosophila* ortholog. Even if an insect species is not amenable to functional analysis, experiments can be done in *Drosophila* to characterize gene function. Finally, stock centers provide fly lines to investigators at minimal cost (e.g., the Bloomington Stock Center, Indiana), and the fly community leads in the use of open source databases (FlyBase and Berkeley *Drosophila* Genome Project, 75). This amassed wealth of information for *Drosophila* serves as a jumping-off point for functional studies in other insect species and for comparative analyses that drive the evo-devo field.

**RNA interference (RNAi):** the use of double-stranded RNA as a tool to knock-down gene expression to study a gene's function

## Tools to Study Gene Function in Other Insects

Molecular and genetic approaches have been used successfully in several insect species to date, including emerging model systems such as *Tribolium castaneum*. Through the development of versatile transposon-based vectors (e.g., 40), transgenics has been extended successfully to other insects, such as drosophilids, mosquitoes, butterflies, moths, and beetles. The UAS/GAL4 system is being tested in *Tribolium* for targeted misexpression (92). More broadly, in situ hybridization and immunohistochemical staining techniques have been established for examining gene expression in developing embryos in many different insects (e.g., 84). Over the past several years, RNA interference (RNAi) in insects has become a powerful and popular genetic tool (and has been reviewed in detail elsewhere, 7). Notably, RNAi was first demonstrated to be successful in *Tribolium* targeting the *Hox* genes *Deformed* (12) and *maxillopedia* (*Tribolium proboscipedia*) (13), and has been used successfully to study the function of *Hox* and other early developmental genes in many insects (7). Because expression pattern is not always indicative of function (e.g., 47), the ability to carry out functional studies in insects other than *Drosophila* was a turning point in evo-devo for comparative analysis of *Hox* gene function. In the following sections, we focus on selected *Hox* evo-devo case studies that have included approaches to link changes in expression pattern and/or protein sequence to functional variation.

## INSECT MODELS AS SYSTEMS TO STUDY DIVERGENCE OF RAPIDLY EVOLVING *HOX* GENES

Insects provide an excellent system for studying the evolution of large gene families (such as the *Hox* genes) because of the extreme diversity of taxa in nature that can be sampled. For example, while most *Hox* genes display conservation in sequence, expression, and function across insects, a few genes in the insect *Hox* cluster are more rapidly evolving. Here we discuss the three well-documented examples of rapidly evolving *Hox* genes and show how changes in both protein and regulatory sequences have contributed to functional switches.

## Just "Ftz-ing" Around During Insect Evolution

The case of *fushi tarazu* (*ftz*) provides a compelling example of a *Hox* gene that has changed function during evolution. *ftz* is thought to have arisen as a duplication of a homeotic *Antennapedia*-like ancestor sometime around the protostome-deuterostome split, and is orthologous to lophotrochozoan *Lox5* (108) (**Figure 1**). *D. melanogaster ftz* (*Dm-ftz*) is a pair-rule segmentation gene expressed in a striped pattern in the primordia of alternate body segments, which fail to develop in *ftz* mutants (39, 56, 79, 116). This pair-rule segmentation function of *Dm-ftz* differs markedly from the function of neighboring *Hox* genes, which are homeotic and specify the identity of body regions.

Changes in both expression pattern and protein sequence have contributed to the switch in *ftz* function from its presumed ancestral homeotic state to its pair-rule segmentation function in *Drosophila*. *ftz* is expressed in stripes in several holometabolous insects and one basal insect

(11, 24, 39, 43, 49, 68), but retains *Hox*-like expression in chelicerates, myriapods, and some crustaceans (44, 48, 51, 83, 108), suggesting that a striped expression pattern was acquired during insect evolution. In addition, changes in the Ftz protein sequence have contributed to the pair-rule segmentation function in *Drosophila*. *Dm*-Ftz interacts with the orphan nuclear receptor Ftz-F1 (38, 127) and together they activate downstream target gene expression to promote segment formation (28, 38, 46, 127, 128). The interaction between Ftz and Ftz-F1 is dependent on a nuclear receptor coactivator-like LXXLL motif in Ftz that binds the AF-2 domain of Ftz-F1 (94, 106, 128). We examined the homeotic and segmentation potential of Ftz proteins from the beetle *Tribolium castaneum* (*Tc*-Ftz) and the grasshopper *Schistocerca gregaria* (*Sg*-Ftz) by ectopic expression in *Drosophila* (43, 61, 62). Antenna-to-leg transformations were seen with both *Tc*-Ftz and *Sg*-Ftz but not *Dm*-Ftz, suggesting that the beetle and grasshopper proteins retain homeotic potential, whereas *Dm*-Ftz lost the potential to carry out homeotic functions even when expressed in a homeotic fashion (**Figure 2**). *Tc*-Ftz also displayed segmentation potential similar to that of *Dm*-Ftz, whereas *Sg*-Ftz showed only marginal segmentation potential (**Figure 2**). These functional properties correlate with cofactor interaction motifs: *Dm*-Ftz lacks the YPWM motif present in most Hox proteins that mediates interaction with Exd, has an LXXLL motif required for Ftz-F1 interaction, and displays only segmentation potential; *Tc*-Ftz has both YPWM and LXXLL motifs and homeotic and segmentation potential in vivo; *Sg*-Ftz has only a YPWM motif and exhibits mostly homeotic potential. We further mapped these two functional motifs spanning ∼400 million years of arthropod evolution and found that while the LXXLL motif was stably acquired once in holometabolous insects, the YPWM motif has degenerated independently in multiple lineages and confers varying degrees of homeotic potential to *Dm*-Ftz (44).

In conclusion, *ftz* switched from a *Hox*-like to pair-rule segmentation gene in insects because of changes in *ftz* sequence and expression. Although functional studies in more insects are needed to study biological roles of diverse Ftz proteins, *Drosophila* has provided an excellent system for testing hypotheses about sequence changes required for a Hox protein to switch roles during evolution.

### *Hox3/zen* Divergence and Its Co-option into a Role in Extraembryonic Membrane Formation

*zen* is another rapidly evolving homeotic gene. It diverged in function from its *Hox3* homolog via changes in both expression and protein sequence, leading to a new role in extraembryonic membrane formation (81). *zen* retains *Hox*-like expression in arthropods such as chelicerates (1, 21, 109), myriapods (48, 51), a crustacean (83), and a basal insect (49) but is expressed earlier than other *Hox* genes during embryogenesis in many insects, in the developing amnion and serosa (93, 113) (summarized in **Table 1**). Despite differences in extraembryonic membrane formation between insects that retain separate amnion and serosal membranes and those with a fused amnioserosa (29, 93), *zen* is expressed in these developing membranes in most insects examined (23, 24, 26, 49, 82, 87, 90, 91, 113) (**Table 1**). Importantly, RNAi studies confirmed that *zen* is required for extraembryonic membrane formation in diverse insects (80, 82, 87, 113).

Zen has also changed at the protein level, as Zen homologs tend to have shorter coding regions upstream of the homeobox and more introns than Hox3 homologs, and many Zen coding regions lack YPWM motifs (81). We found a similar situation with Ftz, in which the YPWM motif has degenerated in many insect lineages (44). Interestingly, all Zen orthologs isolated from insects, except *Thermobia domestica* Zen, lack the YPWM motif (81); we also found that *T. domestica* Ftz has maintained this motif (44).

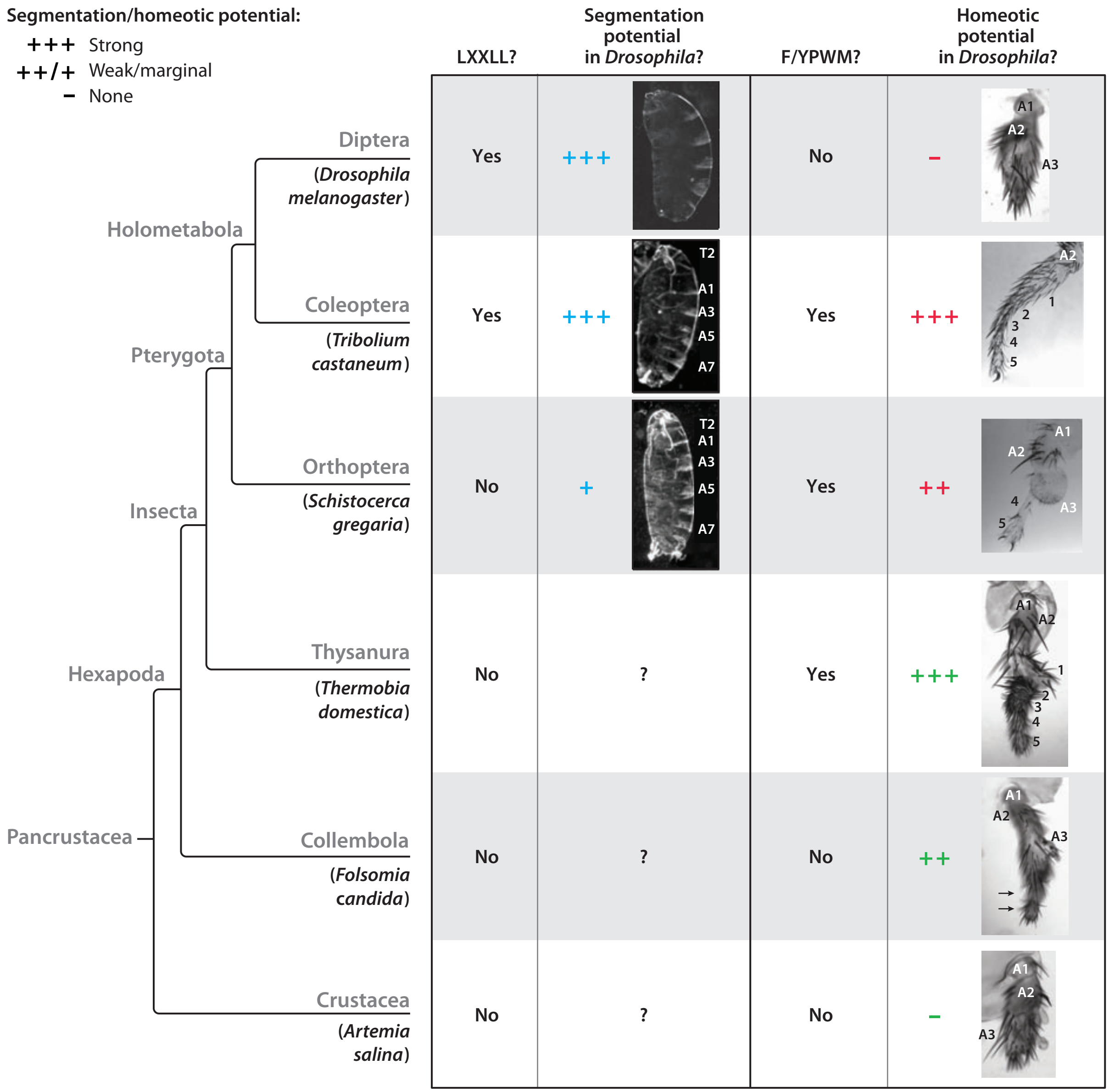


**Figure 2**

Changes in Ftz protein sequence contributed to its switch from homeotic to pair-rule segmentation function in *Drosophila*. Ftz proteins from *Drosophila melanogaster*, *Tribolium castaneum*, or *Schistocerca gregaria* were misexpressed in *D. melanogaster* to determine functional potential. The presence or absence of the segmentation LXXLL motif and homeotic YPWM motif in Ftz proteins is indicated. Ftz proteins were expressed in *Drosophila* with an NGT40-Gal4 driver (*blue plus signs*) or *Dll*-Gal4 driver (*red plus or minus signs*), resulting in segmentation or homeotic phenotypes, as indicated. Functional studies that changed the degenerate F/YPWM (FNWS) sequence in *Drosophila* Ftz to the degenerate motifs found in *Thermobia domestica*, *Folsomia candida*, or *Artemia salina* Ftz proteins are shown in green (plus signs or minus sign), along with their homeotic potential. Segmentation and homeotic potential were scored as strong (+++, complete loss of half of the segments or transformation to a complete leg), weak or marginal phenotype (++/+, not a complete loss of segments or incomplete leg formation), or no phenotype (–, similar to the wild-type situation). Leg and antenna images reprinted with permission from References 44 and 62.

**Table 1 A new *zen* expression pattern in insects allowed for co-option into an early developmental pathway involved in extraembryonic membrane development**

| | Zen sequence | | Zen expression | | |
|---|---|---|---|---|---|
| **Scientific name (References)** | **YPWM** | **Homeodomain** | ***Hox*-like** | **Serosa** | **Amnion** |
| *Archaearanea tepidariorum* (1) | + | + | + | | |
| *Glomeris marginata* (51) | + | + | + | | |
| *Daphnia pulex* (83) | + | + | + | | |
| *Folsomia candida* (81) | + | + | ? | ? | ? |
| *Thermobia domestica* (49, 81) | + | + | + | − | + |
| *Schistocerca gregaria* (23) | − | + | − | + | − |
| *Oncopeltus fasciatus* (82) | − | + | − | + | − |
| *Tribolium castaneum* (26, 113) | − | + | − | + | + (only *zen2*) |
| *Apis mellifera* (24) | − | + | − | + | + |
| *Megaselia abdita* (87, 101) | − | + | − | + | − |
| *Drosophila melanogaster* (90) | − | + | − | + (amnioserosa) | |

In sum, like *ftz*, *zen* is a divergent *Hox* gene that has been co-opted for an earlier embryonic function in insects. It would be interesting to know the function of *zen* in the basal insect *Thermobia*, where it has both *Hox*-like and extraembryonic expression patterns, as this would further elucidate when *zen* acquired a non-*Hox* function and reveal whether it retained ancestral *Hox*-like functions while taking on new biological roles.

## *bcd* Duplication and Neofunctionalization in Dipterans

Arguably the most widely studied rapidly evolving *Hox* gene is *bicoid* (*bcd*), which is required for head development in higher dipterans. *bcd* arose as a tandem duplication of *Hox3/zen* in cyclorrhaphan flies within the past 140 million years, where it has rapidly neofunctionalized (63, 101, 102). *Drosophila* Bcd is required for proper head development, functioning as a classic morphogen to instruct embryonic development via an anterior-to-posterior concentration gradient (33). Although *bcd* is crucial for *Drosophila* head development, the gene is absent from most insects that use different head-patterning mechanisms; thus, Bcd took over a role in higher flies previously carried out by different genes (63, 74). The derived role of Bcd required acquisition of a novel expression pattern—a complex anterior-to-posterior gradient—and changes in protein sequence, conferring novel DNA- and RNA-binding activities (41, 78).

*bcd* functional experiments in cyclorrhaphans provide a glimpse into how rapidly this gene is evolving. Bcd sequences from *Drosophila pseudoobscura* and *D. melanogaster* (species separated by ~25 my) are approximately 86% conserved, but the *D. pseudoobscura* gene and flanking regulatory sequences only partially rescue *D. melanogaster bcd* mutants, due at least in part to improper *bcd* RNA localization (97). *bcd* coding sequences from *Lucilia sericata* and *Calliphora vicina* (blow flies) did not rescue *Drosophila bcd* mutants, suggesting changes in amino acid sequence have species-specific functional differences as well (36). Finally, isolation of the *bcd* locus from *Musca domestica* revealed that *bcd cis*-regulatory regions have diverged considerably from *D. melanogaster* (98).

Like *ftz* and *zen*, *bcd* is a rapidly evolving *Hox* gene in insects. The *bcd* case is unique in that the duplication event giving rise to *bcd* was fairly recent, allowing for easier tracking of evolutionary changes. Examination of *bcd* genes from more cyclorrhaphans may provide further insight into evolutionary constraints at the *bcd* locus.

Together, isolating gene sequences and examining expression and function of these three *Hox* genes in insects have contributed to our understanding of molecular evolution. These studies in particular highlight the fact that flexibility is permitted in both expression and function of a highly conserved set of embryonic regulatory genes. As the number of insects available for study continues to increase, so will our knowledge about these, and possibly additional, rapidly evolving *Hox* genes.

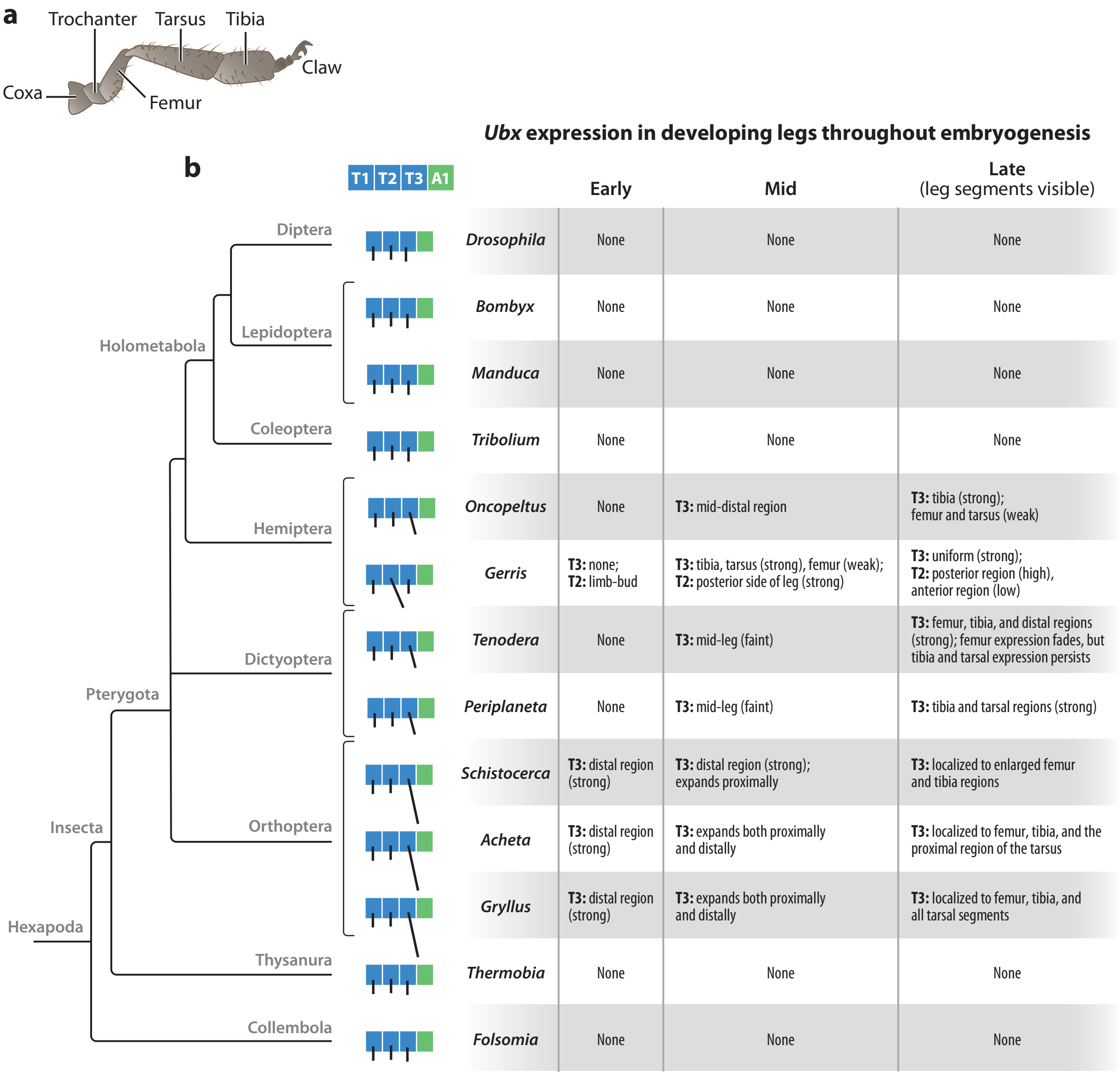
## MORPHOLOGICAL NOVELTIES RESULTING FROM CHANGES IN *HOX* GENES

Despite the conservation of *Hox* genes, there is a great morphological diversity of body plans in metazoans, including insects. This diversity includes variation in wing shape, leg size, and other morphologies unique to different insect families. Although sequence and expression of *Hox* genes have been examined in many insects, only a handful of studies have shown how changes in *Hox* expression, or in their target genes, have contributed to morphological differences. Here, we discuss a few compelling examples that show how variation in a *Hox* gene has promoted morphological evolution and affected body plan diversification.

### The Case of *Ubx* and Leg Morphology

All insects have a pair of legs on each of the prothoracic (T1), mesothoracic (T2), and metathoracic (T3) segments (**Figure 3**). Despite this, there is great diversity in insect leg morphology. Some insects have three uniform pairs of legs (e.g., 67), whereas in other insects, one pair of legs is longer than the other legs. These differences are thought to have evolved as locomotory adaptations. Studies of the developmental basis for these variations revealed a strong correlation between leg length and expression of the *Hox* gene *Ultrabithorax* (*Ubx*). In species with differences in leg pair length, variation in both the timing and domain of expression of *Ubx* during early development was found between elongated legs and nonelongated legs (**Figure 3**). Mahfooz et al. (66, 67) reported that during embryogenesis of several orthopterans and dictyopterans, *Ubx* expression is specifically localized to the leg segments that are longer than other leg segments in the nymph and adult. For example, in nymph grasshoppers, the femur and tibia segments of the T3 jumping leg are enlarged relative to other leg segments, corresponding to the regions where *Ubx* expression was detected in the embryo. Crickets have a hindleg similar to that of grasshoppers, but the tarsal segment is also elongated relative to the other legs. This leg morphology is reflected by differences in *Ubx* expression: Crickets showed *Ubx* tarsal staining, whereas grasshoppers did not. Similar expression patterns were also seen in mantis and cockroach T3 legs, which are somewhat elongated; in dictyopterans this corresponded to *Ubx* expression later in embryogenesis, suggesting that timing of *Ubx* expression is also important in determining leg length. Together, these studies correlate increased *Ubx* expression with increased leg growth, suggesting that changes in *Ubx* expression promoted morphological diversification.

Studies in water striders (hemipterans) analyzed expression as well as function of *Ubx*. In these organisms, the T2 leg is much longer than the T1 and T3 legs. Khila et al. (54) found that during early embryogenesis *Ubx* was expressed in the T2 leg but not the T3 leg. Later in development, *Ubx* was also strongly expressed throughout the developing T3 leg. *Ubx*-RNAi revealed that *Ubx* has opposing functions in the T2 and T3 developing legs: First, *Ubx* promotes growth of the T2 leg, as knocking-down gene expression resulted in shorter T2 legs. Second, *Ubx* shortens the T3 leg, for embryos had a much longer T3 leg when *Ubx* was depleted. In conclusion, many studies have correlated changes in the timing or domain of Ubx expression with variation in leg morphology, and while these changes are sometimes subtle, they likely have adaptive significance.



***Ubx* expression in developing legs throughout embryogenesis**

| | Early | Mid | Late<br>(leg segments visible) |
|---|---|---|---|
| ***Drosophila*** | None | None | None |
| ***Bombyx*** | None | None | None |
| ***Manduca*** | None | None | None |
| ***Tribolium*** | None | None | None |
| ***Oncopeltus*** | None | **T3:** mid-distal region | **T3:** tibia (strong);<br>femur and tarsus (weak) |
| ***Gerris*** | **T3:** none;<br>**T2:** limb-bud | **T3:** tibia, tarsus (strong), femur (weak);<br>**T2:** posterior side of leg (strong) | **T3:** uniform (strong);<br>**T2:** posterior region (high), anterior region (low) |
| ***Tenodera*** | None | **T3:** mid-leg (faint) | **T3:** femur, tibia, and distal regions (strong); femur expression fades, but tibia and tarsal expression persists |
| ***Periplaneta*** | None | **T3:** mid-leg (faint) | **T3:** tibia and tarsal regions (strong) |
| ***Schistocerca*** | **T3:** distal region (strong) | **T3:** distal region (strong); expands proximally | **T3:** localized to enlarged femur and tibia regions |
| ***Acheta*** | **T3:** distal region (strong) | **T3:** expands both proximally and distally | **T3:** localized to femur, tibia, and the proximal region of the tarsus |
| ***Gryllus*** | **T3:** distal region (strong) | **T3:** expands both proximally and distally | **T3:** localized to femur, tibia, and all tarsal segments |
| ***Thermobia*** | None | None | None |
| ***Folsomia*** | None | None | None |

**Figure 3**

Variations in *Ultrabithorax* (*Ubx*) expression in developing insect legs have contributed to morphological diversity. (*a*) A schematic showing the five segments and claw of an insect leg. (*b*) (*Left*) Examples where shifts or variation in *Ubx* expression is correlated with leg diversity. The thoracic segments (T1, T2, T3) (*blue*) and first abdominal segment (A1) (*green*) of arthropods are shown, with relative leg lengths. (*Right*) *Ubx* expression during early, mid, and late embryogenesis correlates with differences in leg morphology seen in nature and is depicted on the left. Data presented in this figure were compiled from References 54, 66, and 67.

## Many Ways to Make a Wing: The Role of *Ubx* in Wing Development

Most modern insects have two pairs of wings on the thorax: one pair on the T2 segment and one pair on the T3 segment (4). Insects such as dragonflies and damselflies have two pairs of similar wings, reminiscent of the ancestral state of winged insects (121); however, wing-pair morphology differs in many extant insects. In most insects, *Ubx* is expressed in the developing T3 segment, where it affects hindwing morphology (17, 59). *Drosophila* and other dipterans have two sets of wings that differ in appearance from one another: The sets of wings found on T2 are important for flight, while the hindwings have been modified to a balancing structure called a haltere. In *Drosophila*, loss-of-function *Ubx* mutations result in transformation of T3 toward T2, with the haltere transformed toward forewing (59). Conversely, mutations that cause ectopic expression of *Ubx* in the developing wing transform wing tissue into haltere tissue (34). These results suggest that *Dm*-Ubx suppresses forewing development. Weatherbee et al. (120) found that Ubx indeed negatively regulates target genes involved in forewing formation, such as *wingless*, *spalt-related*, *vestigial*, *Serum Response Factor*, and *achaete-scute*, by binding *cis*-regulatory regions of forewing-promoting genes such as *spalt*, resulting in silencing of gene expression in the haltere (120).

In Lepidoptera, forewings and hindwings differ, but in contrast to the dipteran haltere, the hindwing is fully developed. Although *Ubx* is expressed in the developing hindwing in the butterfly *Precis coenia* (118), it does not repress the forewing-promoting genes, suggesting target genes have lost *Ubx*-responsiveness in this species (121). In Coleoptera, the T2 and T3 segments have wings, but unlike most insects, the wings on T3 resemble typical hindwings used for flight, while the T2 wings are modified to sclerotized wing covers called elytra (111). RNAi targeting *Tribolium Ubx* (*Ultrathorax*, *Utx*) transformed the hindwing to an elytron (111). Several genes have different expression patterns in the T2 elytron and T3 hindwing, all of which were altered in *Ubx* RNAi experiments (111). These results suggest that *Ubx* functions in the beetle to promote the development of hindwings by repressing genes involved in elytra formation—a taxon-specific role that required reorganization of gene regulatory connections. In sum, *Ubx* shares a role in hindwing development in diverse insects, but its specific role in this process can change.

## Variations in *Scr* and Novel Morphologies on the T1 Segment

*Sex-combs reduced* (*Scr*) is another highly conserved *Hox* gene; it is expressed in the head and thoracic regions of insects during embryogenesis (4). Because *Scr* patterns the T1 segment in insects, it has been suggested that changes in *Scr* contributed to T1 morphological diversity.

**Helmet appendages on treehoppers.** Treehoppers are hemipterans with large, unique helmet structures that protrude from the insect body. Prud'homme et al. (86) examined the evolutionary origin of helmets and found them to be T1 dorsal appendages, much like appendages found on T2 and T3, with flexible attachment points to the body. They further found that transcription factors required for wing formation were expressed in the developing helmet in patterns similar to those seen in the developing *Drosophila* wing, suggesting that similar genetic networks underlie the formation of wings and helmet structures. If the helmets are wing-like structures, *Scr* must not be suppressing wing development in T1 in treehoppers as it does in other insects (18). However, in treehoppers Scr protein was detected throughout T1, where the helmet was developing. Ectopic expression of *Drosophila Scr* or treehopper *Scr* in the developing wing of *Drosophila* produced identical phenotypes, namely suppression of wing growth. Thus, treehopper *Scr* retains the ability to repress wing formation, but it fails to suppress helmet formation. This suggests that Scr-downstream target genes have become unresponsive to Scr in the treehopper T1 segment, possibly due to changes in target gene *cis*-regulatory elements. Recently, Miko et al. (76) questioned the homology of helmet to wing based on detailed morphological comparisons. Irrespective of how this disagreement is resolved, the incredible morphological diversity of treehopper helmets and the rapid progress in identifying the patterning genes controlling its development make this an exciting system for working out molecular mechanisms that lead to development and differentiation of complex and evolutionarily plastic body structures.

**Pronotal horns on beetles.** Many species of beetles also have a unique morphological structure—a pronotal horn—on T1. Moczek and colleagues (119) have studied the origin and diversity of beetle horns in two *Onthophagus* species that exhibit differences in both size and location of the T1 horn. They found that *Scr* expression was consistent with that of other winged insects, suggesting variation in *Scr* expression does not explain horn diversity. However, RNAi studies revealed that in addition to "traditional" homeotic transformations (119), *Scr*-RNAi led to variation in T1 horn size. These animals also showed reduction in pupal horn size, which varied between sexes in one species. Adult horns also exhibited both size- and sex-specific growth reductions, but these differences were seen in the opposite species during pupal growth (i.e., the species that showed no sex-specific differences in pupal pronotal growth showed significant size differences in adult horns). While it has been shown that programmed cell death is important in determining horn size and position (55), the mechanism through which *Scr* acts to promote T1 horn formation is not known. It is likely that differential regulation of Scr target genes plays a role in the tremendous diversity in beetle horn size in nature. In sum, *Scr* has retained a highly conserved role in patterning the labial segment and T1 legs in most insects but has taken on new roles important for the diversification of species-specific adaptations of this segment.

## CONCLUSIONS

*Hox* genes are important for patterning the body of metazoans, and changes at many different levels have been implicated in switches in gene function and acquisition of novel gene functions. Changes in protein sequence, like those in Ftz, allowed for changes in protein cofactors, co-opting Hox proteins into new developmental pathways. Changes in *Hox* expression domains, like novel expression of *zen* in extraembryonic membranes and *Ubx* expression variation in leg development, have allowed *Hox* genes to acquire new functions. Finally, changes in the downstream target genes activated by Hox proteins are important in the formation of novel morphologies seen in insects, such as butterfly wings and beetle horns. Insects continue to serve as model systems so that scientists can study how changes in genes fundamental to body plan development contribute to diversity in nature. Though *Hox* genes are only one set of genes in an organism's genetic toolkit, insects have proved to be an effective system for studying how changes in these important developmental genes have contributed to morphological evolution. Future studies, on *Hox* and other developmental genes, will contribute to our understanding of evolution and the field of evo-devo as a whole.

### SUMMARY POINTS

1. All metazoans possess a genetic toolkit of regulatory genes important for embryonic development. These genes are highly conserved, raising questions about how morphological diversification can be promoted by a shared set of regulators.
2. Insects are excellent models for examining evo-devo questions. Powerful genetic approaches in *Drosophila* and RNAi in nonmodel species allow comparative studies in diverse insects, reflecting hundreds of millions of years of evolutionary divergence.

3. *Hox* genes, classic toolkit genes important for determining segment identity, were first discovered in *Drosophila* in the 1980s. *Hox* function has now been studied in many insects, including other dipterans, hymenopterans, coleopterans, hemipterans, orthopterans, dictyopterans, and thysanurans. Variation in *Hox* gene spatiotemporal expression and protein function during development generates morphological diversity in nature.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**130. When expressed in *Drosophila*, *Hox-a5*, a mouse homolog of Scr, recapitulated Scr homeotic functions, which were shown to require the YPWM motif.**

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# Contents

## Indexes

## Errata

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