Abstract
Evolutionary developmental biology (evo-devo) is a recently established discipline that connects evolutionary theory with developmental biology. However, despite evo-devo’s integral use of diverse insect taxa as model systems and its interdisciplinary approach, current introductory entomology textbooks fail to fully integrate evo-devo into the undergraduate curriculum. We argue that an evo-devo case-study-based approach, focused on adult development, will not only familiarize students with exciting findings in this field, but will also help them deepen their understanding of basic entomological concepts. After a short background of the most important findings and methods currently used in evo-devo, we outline five case vignettes that span a variety of insect groups and entomological topics, including morphology and sexual selection.

Key Words: entomology, evolutionary developmental biology, evo-devo.

Introduction
Evolutionary developmental biology, popularly known as “evo-devo,” is a recently established discipline that attempts to connect developmental processes with evolutionary theory (Raff, 2000). Although developmental biology and embryology were foundational to early evolutionary thinkers (e.g., with Ernst Haeckel’s famous but flawed biogenetic law that “Ontogeny recapitulates phylogeny”; Gould, 1977), the rise of population genetics and the subsequent establishment of the “modern evolutionary synthesis” (Huxley, 1942) led to an essentially complete exclusion of development in our understanding of evolution. Despite the enormous success of the modern synthesis, it became clear by the 1970s that a full understanding of evolution could be achieved only with the reintegration of development into evolutionary theory, because it is the proximate mechanism that forms phenotypes upon which natural selection acts (Gould, 1977). However, given the limited number of existing molecular tools at that time, the successful unification of evolution and development—which Charles Darwin, Haeckel, and others envisioned—had to wait for almost two more decades, when major technological advances in molecular biology allowed scientists to begin uncovering the genetic underpinnings of plants and animals (Raff et al., 1999).

Important molecular breakthroughs included polymerase chain reaction (PCR) and DNA sequencing, imaging techniques of gene and protein expression patterns (e.g., in situ hybridization and immunolocalization), as well as tools that allowed researchers to alter gene expression in a wide variety of organisms (e.g., RNA interference, or RNAi). While many of these tools were originally established in insect model systems such as the fruit fly Drosophila melanogaster and the flour beetle Tribolium castaneum, they have also been applied successfully to more morphologically interesting species, such as the aposome milkweed bug Oncopeltus spp. (e.g., Angelini & Kaufman, 2005), the African butterfly Bicyclus anynana (Beldade & Brakefield, 2002), the horned dung beetles of the genus Onthophagus (e.g., Kijimoto et al., 2012), as well as the charismatic rhinoceros beetle Trypoxylus dichotomus (Emlen et al., 2012) and luminescent fireflies in the genus Photuris (Stansbury & Moczek, 2014).

In response to the considerable success of such studies, current introductory undergraduate entomology textbooks, such as the rightfully popular The Insects: An Outline of Entomology (Gullan & Cranston, 2014), have begun to dedicate special box-insets or short paragraphs to evo-devo, noting that “more detailed information on the genetics of morphological evolution are beyond the scope of an entomology textbook” (Gullan & Cranston, 2014). However, we argue that a more effective and efficient way of introducing evo-devo can be achieved by fully integrating its findings throughout the course, instead of dedicating a single lecture to it, thus emphasizing the interdisciplinary approach of evo-devo.
In our experience, this is best done in the form of case studies, some of which we will present at the end of this article. Such an approach not only allows students to apply important molecular techniques to real-world problems, but it also illustrates that developmental modifications are the proximate underlying mechanism of phenotypic change across generations. In addition, we argue that the traditional focus on embryonic development, as found in the aforementioned textbook, while important, might hinder the ability of students to fully grasp the importance of the ontogeny of familiar anatomical traits. Instead, we suggest a shift in focus toward later developmental stages of macroscopic adult traits, such as wings, legs, and spectacular structures evolved by sexual selection (e.g., enlarged mandibles of stag horn beetles), to provide students with a more accessible and relevant context in which to study evo-devo and to concurrently enhance their understanding of entomology.

In this review, we will outline some of the major findings of evo-devo, which will be followed by a simple introduction to the most important molecular tools currently used in this field. Lastly, we will include several case studies in which students generate testable hypotheses and apply the molecular tools to predict possible outcomes based on real data, all of which were used in an introductory entomology course at Fairleigh Dickinson University (Madison, New Jersey) in the fall semester of 2015.

**Major Insights of Evo-Devo**

Evo-devo, or the application of developmental genetics to evolutionary questions, has contributed significantly to our current understanding of animal and plant forms (Carroll et al., 2004). Evo-devo attempts to apply the principles and molecular tools of developmental genetics to answer proximate evolutionary questions, or the “how” questions in evolution. For example, while traditional evolutionary theory focused on the selective environmental pressures and other influences that resulted in populations of giraffes with increasing neck lengths, evo-devo asks, “What specific genetic modifications cause giraffe neck vertebrae to lengthen, in comparison to their ancestors, during embryogenesis?” This approach allows us to investigate a wide variety of previously less tractable problems, including changes in developmental gene regulation (Carroll, 2008), the origin of novelties (Moczek, 2008; Stansbury & Moczek, 2014; Wagner, 2015), the notion of developmental influence and constraint upon evolutionary trajectories (Beldade & Brakefield, 2002; Raff, 2012), the multilayered definition of homology (Wagner, 2015), and the role of developmental plasticity in evolution (Moczek et al., 2011). Given the limitations of the modern synthesis (Huxley, 1942) to answer such questions (Laland et al., 2015; but see Hoekstra & Coyne, 2007), prominent researchers of this field proposed an expansion, dubbed the “extended evolutionary synthesis” (Pigliucci & Mueller, 2010) to account for the creation of new phenotypes through changes at the developmental genetic level.

One of evo-devo’s most important and surprising contributions to date was the revelation of extensive conservation of the genes directing the development of very distantly related animal taxa (see below). Here, it became clear that much of the regulation of metazoan development is controlled by relatively few genes. The protein products of this “genetic toolkit” (Carroll et al., 2004) act as top-level executives, activating or inhibiting expression of a wide variety of downstream genes by either binding to regulatory sequences in DNA (as transcription factors) or diffusing through embryos to activate signaling cascades in target cells (as signaling factors/morphogens).

Among the first of the toolkit genes discovered were those that determine regional identity along the anterior-posterior axis. These were initially termed “homeotic” genes, because mutants exhibited dramatic transformations of segment identity. For example, mutations in the *Abdominal-B* (*Abd-B*) gene result in flies with legs in place of genitalia (e.g., Estrada & Sánchez-Herrero, 2001), while loss-of-function mutations of the *Ultrabithorax* (*Ubx*) gene cause the fly to develop a pair of wings instead of halteres on the metamorphic segment (Lewis, 1978; for a case study on this gene, see below). Each of the eight homeotic genes discovered in *D. melanogaster* was found to contain a conserved, 180-base-pair region that researchers termed the “Homeobox” ultimately lending the name “Hox” to this group of genes. The Homeobox region is translated into the highly conserved, 60-amino-acid homeodomain, a DNA binding site that allows the Hox proteins to function as transcription factors that regulate the expression of a wide variety of downstream genes in a region-specific fashion. The role of each Hox gene is thus to impart a specific identity to a segment (or, sometimes, a group of adjacent segments). For example, while both the meso- and meta-thoracic segments form wing discs in fly embryos, these primordial cells have dramatically different developmental fates. The *Antennapedia* (*Antp*) Hox protein activates a suite of target genes that cause the mesothoracic disc to differentiate into fully fledged wings (Hughes & Kaufman, 2002b). The *Ubx* protein, expressed in the metathoracic segment, represses some of the same target genes, inhibiting the growth of wings, and activates other targets that promote the formation of halteres (Lewis, 1978). Interestingly, and for reasons that remain incompletely understood, Hox genes are not only found clustered together on chromosomes, but also are expressed along the anterior-posterior axis in the same order in which they are linearly arranged on the chromosome. Ed Lewis (1978), who made this discovery, coined the term *colinearity* for this unique organization of Hox genes. Strangely, subsequent studies found that the same basic set of Hox genes are present in all Bilateria (including humans), and that both their colinear chromosomal arrangement and their function in the specification of the anterior-posterior axis are conserved (Carroll et al., 2004). Evo-devo therefore demonstrates a remarkable unity in the construction of all animal bodies with Bilaterian body plans, despite their extraordinary diversity, an invariably fascinating fact to students of entomology.

While Hox genes are important in determining the fates of segments, the formation of structures within segments is governed more directly by a diverse set of field-specific selector genes (Carroll et al., 2004). Like Hox genes, these genes function as regulatory signals, activating and deactivating downstream gene expression via the DNA binding motifs of their protein products (Carroll et al., 2004). These DNA binding domains come in a variety of forms, such as leucine zippers, zinc fingers, homeodomains, and others (note: while the homeodomain is a common DNA binding domain found in many regulatory proteins, the designation “Hox gene” is reserved for the canonical group of clustered genes responsible for anterior-posterior axis specification; Carroll et al., 2004). Field-specific selector genes regulate the development of a wide variety of traits, such as eyes (eyeless gene; Halder et al. 1995), length of appendages (Distal-less,
Panganiban et al., 1997), and flight appendages (vestigial and scalloped; Weatherbee & Carroll, 1999). Given the observable morphological variation along the proximodistal axis in insect appendages for beginning entomologists, we suggest that Distal-less is an especially intriguing gene to develop case studies around.

Just as Hox genes show a remarkable degree of conservation across metazoans, some field selector genes are so evolutionarily stable that they are actually interchangeable across incredible phylogenetic distances (Carroll et al., 2004). The evolutionary stasis of the homeobox in eyeless famously allowed the Swiss developmental biologist Walter Gehring to use the human copy of the Drosophila gene eyeless, known to vertebrate researchers as PAX-6, to phenotypically rescue eyeless fruit fly mutants, which otherwise would not develop eyes at all (Gehring & Ikeo, 1999). Additionally, the ectopic expression of this gene in other regions than the fly head has been shown to lead to the development of ectopic eyes in antennae, legs, and wings (Halder et al., 1995), demonstrating the executive nature of selector gene function.

While other genes of the genetic toolkit have been described (compartment selector genes and cell-type-specific selector genes), we recommend the development of case studies focusing on Hox and field-specific selector genes, because changes in their expression usually lead to profound macroscopic changes in the phenotype, which can be used to explain morphological diversity within and between insect groups. It has been suggested that subtle changes in the expression of, or regulatory interactions within, the genetic toolkit (termed cis-regulatory change), as well as the responsiveness of the target genes, are the driving force of major evolutionary evolution (Pick & Heffer, 2012).

Basic Methods of Evo-Devo

Visualizing Patterns of Gene Expression

An emerging theme of evo-devo research is that the evolution of morphology is often a consequence of changes in gene regulation (i.e., changes in the timing and/or spatial positioning of gene network activation; Carroll et al., 2004). This necessitated that researchers were able to visualize patterns of gene expression during development (i.e., in embryos or pupae). Two of the most widely used methods are in situ hybridization and immunolocalization.

Eukaryotic gene expression involves the transcription of a gene sequence from the nuclear chromosome, then nuclear processing of the resulting messenger RNA, followed by exportation of the RNA to the cytoplasm, where translation of the message into a protein occurs. In situ hybridization is intended to detect the presence of specific cytoplasmic mRNA in tissues/tissue sections of interest. This is accomplished by using a specifically designed piece of single-stranded RNA, or “probe,” which serves as a reverse complement to the mRNA of interest. The probe binds stably to the endogenous mRNA transcript through complementary base pairing and is usually labeled with an antigenic molecule to allow for detection by a specific antibody. This antibody is labeled with either a fluorescent molecule or an enzyme that catalyzes a reaction to generate a colored precipitate. Through this procedure, the researcher is able to visually identify areas containing the mRNA of interest, indicating the expression of the gene in a tissue (for a more extensive review of the technique, see Levsky & Singer, 2003).

In immunolocalization experiments, researchers visualize the presence of a protein in tissues via fluorescently labeled antibodies (Carroll et al., 2004). Immunolocalization requires protein-specific antibodies, usually generated by collecting serum from a mammalian host that has been inoculated with the protein of interest. For this reason, the method can be substantially more expensive and time-intensive than in situ hybridization, if an antibody is not already commercially available. Fortunately, as emphasized above, because many developmental regulatory genes are highly conserved in evolution, antibodies generated to detect a protein in one animal can often be used to detect the homologous protein in widely disparate taxa (e.g., an antibody for the Distal-less protein functions in arthropods as well as vertebrates; Panganiban et al., 1997).

We argue that incorporating techniques of gene-expression visualization in entomology classes enhances students’ understanding of biology in a unique way. Specifically, understanding patterns of gene expression can reframe discussions of anatomy and what constitutes a discrete anatomical unit (see case vignette 1 below).

Assessing Gene Functions

While expression studies remain a critical tool in establishing the presence of a gene product in a particular tissue, it is often necessary to evaluate the function of a gene more directly. Gene function is usually analyzed through experimentally disabling a gene of interest and observing any phenotypic consequences that follow – any detectable defects can then be attributed to the induced loss of gene function. In D. melanogaster, sophisticated crosses can be used to delete, or “knockout,” protein-coding, promoter, or regulatory regions of a gene of interest to evaluate various aspects of its function. However, evo-devo research often relies on our ability to perform comparative development genetics, necessitating studies in non-model organisms for which the majority of molecular tools have not yet been developed. These more “exotic species,” which include dung beetles and fireflies, often present life-history challenges that render mutation-based methods of gene inactivation impractical.

Fortunately, the Nobel Prize-winning RNA interference (RNAi) method has dramatically expanded the number of taxa that can be targeted in reverse genetic approaches. RNAi is a cellular pathway that results in post-transcriptional inhibition of gene expression, which is thought to have evolved as a defense against viral genetic elements (Obbard et al., 2009). A full explanation of the complex RNAi pathway is beyond the scope of this paper, but when utilized in research, a double-stranded RNA (dsRNA) molecule is generated whose sequence corresponds to a specific endogenous mRNA. Then this dsRNA is introduced into the organism (usually via injection or feeding) prior to the development of the trait of interest. The dsRNA is taken up by the cells of the organism, where it is cleaved into short fragments that are incorporated into a large protein complex. This complex subsequently degrades any matching endogenous mRNA found in the cytoplasm, effectively interrupting expression of the target gene prior to translation (for a more extensive description of RNAi, see Perrimon et al., 2010). Through this method, any gene can be theoretically inhibited and its effect on the phenotype evaluated. We argue that the incorporation of gene function studies, especially those that explore the genetic basis of charismatic traits, can dramatically enrich
Evo-Devo Case Studies in Entomology

Why Case Studies?

In our experience, memorization of gene function is not sufficient to gain a deep and long-lasting understanding of evo-devo. Instead, we suggest the use of active learning in the form of case studies that allow students to integrate evo-devo research throughout an introductory entomology course. Active-learning strategies are supported by numerous studies (e.g., see meta-analyses in Dochy et al., 2003; Freeman et al., 2014), as well as by the National Center for Case Study Teaching in Science at the University at Buffalo and the U.S. Department of Education, which indicate a high effectiveness of context-based learning when compared to traditional lecture style (e.g., Williams et al., 2007). Importantly, we contend that a case-based approach will not only strengthen the students’ understanding of genetic and evolutionary principles but will also allow them to better retain general entomological material such as basic insect anatomy, which traditionally requires considerable rote memorization.

For example, making successful predictions of the phenotypic effects of downregulating the gene Ubx in Orthopterans requires students not only to understand the principle of RNAi and the function of Ubx, but also requires them to apply their knowledge of the basic morphology of this group (namely the relative elongation of the metathoracic legs; for details, see below). Below, we list a few selected case vignettes, which were used previously in a classroom setting by one of the authors. We suggest that these case studies could be presented within the context of topics like wing evolution, insect anatomy (especially when discussing specific taxa), or sexual selection, which refines understanding and helps to emphasize the interdisciplinary approach of evo-devo. As readers might note, our case studies become increasingly complex, reflecting a deepening understanding of entomology and evo-devo acquired through the progression of a hypothetical course.

Case Vignette 1: Variation in “Evolvability” among Major Arthropod Groups

Background. Insects, the most species-rich taxon known on this planet, are commonly cited as among the most evolutionarily successful animal groups, in both the variety of ecological niches inhabited and the diversity of morphological features exhibited (Grimaldi & Engel, 2005). It has been suggested that understanding the underlying patterns of insect gene expression using the molecular tools of evo-devo may provide a partial explanation for their success.

As discussed above, the Hox genes are a critical director of regional identity along the anterior-posterior axis. A defining feature of insects along this axis is the thorax with its three leg-bearing segments. When gene expression in this region was visualized, it became clear that each thoracic segment expresses a unique Hox gene, or at least a unique combination of Hox genes (Hughes & Kaufman, 2002b). Thus, since the development of each insect thoracic segment is directed by a different set of downstream Hox target genes, the morphology of the individual segments, as well as the appendages they bear, are independently modifiable. This fact might explain, in part, the incredible diversity of insect appendage morphologies and the profound degree of functional subdivision found on adjacent segments within a single insect taxon, such as Mantodea. In contrast to the remarkable diversity of insect appendage specialization, myriapod walking legs are conspicuously uniform in their appearance. Intriguingly, a strikingly different pattern to insects was found when the underlying Hox expression in myriapods was visualized. Here, the many leg-bearing segments are all under the control of the same set of Hox genes (Hughes & Kaufman, 2002a). Therefore, in contrast to insects, a change in the developmental network resulting in modification of a myriapod leg would likely affect all legs in unison, severely limiting the evolutionary flexibility (sometimes referred to as “evolvability”) of the myriapod lineage in relation to their insect cousins (for a more in-depth discussion, see Carroll, 2006; Stansbury & Moczek, 2013).

Case Vignette. We presented this vignette to our students after a discussion of the segmental organization of the arthropod body plan and an introduction to the major arthropod taxa. At this point in the course, the students were generally familiar with the logic of Hox gene function and their colinear organization, but we had not emphasized precisely the pattern of Hox expression within arthropod bodies. To introduce this vignette, we presented the students with specimens that demonstrate the marked heterogeneity of insect legs as well as the uniformity of myriapod legs (e.g., specimens included mantids, orthopterans, and myriapods). We then asked the students to explain why the ability to functionally subdivide appendages on adjacent segments might drastically increase a lineage’s evolutionary capacity to exploit a wide variety of niches. After this discussion, we reminded the students that Hox genes regulate the identity of segments and then asked them to predict the number of Hox genes expressed within the insect thorax and compare this with the number of Hox genes of myriapod locomotory segments. We then revealed the immunolocalization pattern in insects, with each thoracic segment expressing a unique Hox gene (Kosman et al., 2004), and used in situ hybridization images in myriapods to demonstrate that Hox expression is uniform throughout the myriapod leg-bearing region (Hughes & Kaufman, 2002a). We used these predictions to speculate on how these patterns impart developmental flexibility to insect evolution, while possibly impeding the subdivision of appendage morphology in millipedes and centipedes.

Here, evo-devo and methods of gene-expression visualization provide insight into large-scale morphological differences between major arthropod groups and possibly explain, in part, the high success of insects compared with myriapods.

Case Vignette 2: Evolution of Wings

Background. Recent paleontological findings support developmental observations in suggesting that wings evolved through a combination of natal extensions, which contributed the bulk of the wing tissues, and co-option of the pleural elements of the leg, which provided the
necessary articulation and musculature (Niwa et al., 2010; Clark-Hachtel & Tomoyasu, 2016; Prokop et al., 2017).

Paleozoic fossils indicate that, unlike modern insects, species of Palaeodictyoptera and Geroptera bore wings on all three thoracic segments (Figure 1; Kukalová, 1970).

Notably, in these ancient species, the pair of wings on the first thoracic segment (T1) are markedly smaller than those found on the second and third thoracic segment (T2 and T3). It has been suggested that an expansion in size of T2 and T3, which led to a correlated increase of musculature found in these segments, led to the loss of the T1 wing and a corresponding increase in flight ability (Ross, 2017). In fact, many modern insects have evolved various mechanisms to couple hindwing and forewing movement, effectively reducing the separate wings to a single, functionally cohesive apparatus, further increasing flight efficiency (Gullan & Cranston, 2014).

While modern insects do not develop wings on the first thoracic segment, Tomoyasu et al. (2005) showed that RNAi of the Hox gene, *Sex combs reduced* (*Scr*), in the flour beetle *Tribolium castaneum* leads to the development of elytra in T1, demonstrating that *Scr* is an important suppressor of wing development in T1. We suggest presenting students with pictures of *Scr* RNAi individuals (Figure 2), asking them to describe the wing-specific role of *Scr* in the first thoracic segment in flour beetles, and finally allowing them to extrapolate this to (modern) insects in general.

Case vignette. We presented this vignette to our students during a discussion on thoracic anatomy and wing evolution. We provided them with the background information above, including figures showing a reconstruction of a hypothetical Paleozoic insect (Figure 1; Kukalová, 1970) and the phenotypes found by Tomoyasu et al. (2005).

We then asked them to consider and evaluate the following statements:

(a) *Sex combs reduced* (*Scr*) is expressed in the first thoracic segment.

(b) *Scr* is not expressed in the first thoracic segment.

(c) RNAi of *Scr* would lead to a loss of wings in the first thoracic segments of ancestral insects.

(d) The function of *Scr* has changed over the course of insect evolution.

(e) The expression pattern of *Scr* has changed over the course of insect evolution.

In our course, this was the first scenario we used to invite students to apply their understanding of RNAi to a major morphological transition in insect evolution. Many students struggled to find the correct answer, as it required understanding the “negative logic” of RNAi. We used this as an opportunity to reiterate the basics of RNAi. First, the students must understand that RNAi downregulates gene expression and, thus, traits that appear (or disappear) in RNAi-treated individuals are regulated negatively (or positively) by the specific gene in question. Said another way, if a trait does not develop in response to RNAi treatment, it can be concluded that the development of this trait is at least partially initiated by the expression of the RNAi-downregulated gene. On the other hand, if a new trait appears in RNAi individuals, this is strong evidence that the gene in question normally acts to suppress the formation of the trait.

In the case of *T. castaneum*, it was observed that an ectopic trait (wings at T1) developed in individuals in which *Scr* was...
downregulated. Thus, it appears that the expression of this gene causes the suppression of wings at T1, a conclusion that is consistent with the wingless T1 phenotype of all modern insects.

At this point, students were directed to transfer this knowledge to a reconstruction of a Paleozoic insect. These insects bore wings on the first thoracic segment, in contrast to extant taxa, but similar to flour beetles exposed to RNAi-mediated downregulation of Scr. Consequently, students might predict that Scr, which has been shown to inhibit the development of wings on the first thoracic segment, is not expressed in the first thoracic segment of a Paleozoic insect with three pairs of wings. An alternative hypothesis is that the expression pattern of Scr has remained static over insect evolution, but that the gene evolved a novel function during the Paleozoic, namely the ability to suppress “wing-building genes,” which ushered in the transition to winglessness within its expression domain. Expression patterning images, especially from phylogenetically basal insect taxa (e.g., through in situ hybridization) can be used as evidence allowing students to discuss the relative merits of each hypothesis.

**Case Vignette 3: Wing Modifications in Diptera & Coleoptera**

**Background.** As outlined above, most insects develop a pair of wings on both T2 and T3. However, in several insect orders, the forewings and hindwings are dimorphic and thus can be used in a novel behavioral context.

For example, Diptera fully develop only their forewings, while the hindwings develop into halteres, an important balancing organ for flies (Grimaldi & Engel, 2005). Through knockdown and knockout mutations, it has long been known that the expression of the Hox gene, *Ultrabithorax* (*Ubx*), gives identity to the third thoracic segment and its appendages (Lewis, 1978). Consequently, *Ubx* promotes haltere development in flies, and reduced expression of *Ubx* (either through mutations or RNAi) leads to a transformation of the halteres into a second pair of forewings, a phenotype that lends the gene its name. On the other hand, ectopic expression of *Ubx* in the second thoracic segment has been shown to give T2 (and its appendages) a T3 identity (Heffer & Pick, 2013). Thus, flies with such mutations develop a second pair of halteres in place of their forewings. Thus, *Ubx* has been shown to be an important regulator of the morphological forewing and hindwing dimorphism found in flies.

**Case vignette.** We presented our students with this case vignette during a discussion of the orders Diptera and Coleoptera. In the latter, forewings evolved into elytra, while the hindwings retained the flight function, in contrast to dipterans (Gullan & Cranston, 2014). Using the background presented above, we asked the students to predict the effect of *Ubx* RNAi on beetles like *T. castaneum* (Tomoyasu et al., 2005) or the charasmatic dung beetle *Onthophagus* (Wasik et al., 2010). As in flies, *Ubx* is expressed in the third but not in the second thoracic segment in beetles and thus gives the third thoracic segment and associated appendages their unique identity. We presented the students with wild-type pictures of the respective species and used these to compare and contrast forewing and hindwing morphology in beetles and flies. After this discussion, we asked them to predict the phenotype they would expect in *Ubx* RNAi-treated beetles.

As in all insects, *Ubx* serves to regulate T3 identity in beetles and therefore initiates gene expression associated with building the hind flight wings in the group. Thus, just as in *Drosophila*, *Ubx* RNAi in *Tribolium* transforms the third thoracic segment into a second thoracic segment. In contrast to flies, this causes the hindwing to develop into a second pair of heavily sclerotized elytra in beetles (Tomoyasu et al., 2005).

We further asked the students to predict how ectopic expression of *Ubx* in *T. castaneum* might affect forewing morphology (under such conditions, elytra are transformed into hindwings). We used figures from Tomoyasu et al. (2005) to show the students whether their predictions were supported by experimental evidence.

**Case Vignette 4: Evolution of Leg Morphology**

**Background.** In addition to determining the identity of the third thoracic segment, *Ubx* has also been co-opted as an important regulator of relative leg length in a variety of insect species (Heffer & Pick, 2013), including grasshoppers (Orthoptera: *Gryllus* spp.), praying mantises (Mantodea: *Tenodera* spp.), and cockroaches (“Blattodea”: *Periplaneta* spp.). Here, specific regions of the hind leg experience high expression levels of *Ubx* during development, which correspond to an elongation of those regions (Heffer & Pick, 2013). For example, many Orthoptera develop a greatly enlarged femur and tibia of the hind legs, adapted for jumping. Through in situ hybridization, it has been shown that these exaggerated regions of the hind leg show a region-specific increase in *Ubx* expression during development (Heffer & Pick, 2013).

**Case vignette.** This vignette was presented during our discussion on specific insect orders as outlined above. Here, after introduction of Orthoptera, “Blattodea,” and Mantodea, we presented the students with preserved specimens of these groups and asked them to identify the correct order. Afterward, the students were asked to focus on describing the relative contribution of each hind leg segment (coxa, femur, tibia, tarsi) to overall leg length, when compared with the foreleg. This also allowed the students to revisit basic insect leg anatomy and was followed by a discussion on the fitness advantages of hind-leg elongation.

At this point, we presented the students with the background above and asked them to predict the answers to these questions:

(a) Which of the hind-leg segments have increased *Ultrabithorax* (*Ubx*) expression during insect development?

(b) If an *Ubx* RNAi experiment was conducted in each of the species, which of the hind-leg segments would be affected?

(c) Looking at isolated in situ hybridization images from an Orthopteran embryo, which is the fore-leg and which is the hind-leg?

The students were then shown pictures of in situ expression and asked whether their predictions were confirmed by actual research (these images can be found in Mahfooz et al., 2004, 2007).

To remind students of the global influence of *Ubx* in the identity within T3, and to tie in this vignette with previous material, additional questions might include a hypothetical knockdown experiment and predictions of the collective effects on leg and wing development in Orthoptera. Forewings and hindwings in *Gryllus* are overall very similar in appearance, and therefore, while we would expect to observe extreme shortening of the T3 femur and tibia in response to *Ubx* RNAi, we would expect only minimal effects on the hindwings.
Case Vignette 5: Allometric Growth & Sexual Dimorphism

**Background.** Some of the most spectacular examples of sexual dimorphism are found within insects. For example, most males of the dung beetle genus *Onthophagus* develop a wide variety of prominent extrausions (“horns”) on the head and/or thorax, which aid in male–male competition to gain access to females (Emlen, 1997). Other male weaponry found in insects include highly enlarged mandibles (e.g., staghorn beetles such as *Lucanus elaphus* and dobsonflies such as *Corydalus cornutus*), extremely elongated forelegs (e.g., harlequin beetles, *Acrocinus longimanus*), and even huge eyestalks (e.g., flies like *Teleopsis dalmanni*; Emlen & Nijhout, 2000; Emlen, 2014). While all of the above-mentioned traits, with the exception of horns, can be found in both sexes, it has been shown that these traits grow faster in relation to body size in males but not in females. This growth relationship is called *positive allometry* or *hyperallometry* (Figure 3; Shingleton et al., 2007).

In contrast, most other traits grow nearly isometrically with body size, which is possibly caused by functional constraints (Frankino et al., 2005). A few insect traits, most famously male insect genitalia, grow at a slower rate than body size and thus have a *hypoallometric* relationship (Eberhard et al., 1998). As a consequence, males within many insect species develop similarly sized genitalia irrespective of their body size (Eberhard et al., 1998).

While overall adult body size in insects is strongly influenced by nutritional status during development, differential growth rates among traits have only recently begun to be understood (Shingleton et al., 2007; Emlen et al., 2012; Nijhout & German, 2012; Snell-Rood & Moczek, 2012; Shingleton & Frankino, 2014). Recent studies suggest that traits vary in their sensitivity to nutrients found in the hemolymph (Shingleton et al., 2007). Consequently, horns and other hyperallometric traits have been shown to respond more rapidly to nutrients with accelerated growth than traits with an isometric and hypoallometric relationship (Emlen et al., 2012).

The differential growth response to nutrient availability is thought to be mediated by the *insulin signaling pathway* (Figure 4; Carter & Brunet, 2007; Shingleton et al., 2007; Emlen et al., 2012; Eijkelenboom & Burgering, 2013), in which high nutrition levels increase the concentration of insulin-like peptides (ILP) and insulin-like growth factors (IGF) in the hemolymph. Each cell has insulin receptors (InR) found on their cell membrane to which both of these factors (ILP and IGF) can bind and thereby activate. Thus, increased nutrients increase the activation of InR. Activated InR activate AKT through the activation of phosphoinositide 3-kinase or P13K. An increase in AKT leads to a downregulation of FoxO (Forkhead Box, subgroup O), which acts as an important inhibitor of cell proliferation by binding to specific regions of the DNA. Thus, increased nutrition levels lead to an inhibition of FoxO, which results in acceleration of growth rates or cell proliferation (for more details on this pathway, as well as other regulators of cell growth and proliferation in insulin signaling, see Eijkelenboom & Burgering, 2013).

In our experience, students are better able to understand such a diagram when it is presented sequentially. For example, in our lecture, we revealed only the first few components of the pathway, while blocking out the remainder of the diagram. Once the diagram was fully revealed, we mentioned that not all traits respond equally to increased levels of ILP and IGF and asked the students to explain this by using the diagram, focusing on insulin receptors. Most student teams readily suggested that the number of insulin receptors on cells of a given tissue could be important in the regulation of differential growth between organs.

**Case vignette.** This vignette was presented as a case study for sexual selection, and additional topics related to sexual selection (honest signals, parental investment, etc.) were included. (However, we don’t have space in this article to provide the necessary background for

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**Figure 3.** Relationship between (log) body size and the (log) length of three different traits (tibia, genitalia, and horns) in male dung beetles (*Onthophagus gazella*). Each symbol depicts one individual. Regression line indicates different slopes and thus different static allometric relationships. Genital size changes very little with increasing body size (hypoallometry), whereas horns exhibit hyperallometric growth, which is commonly found among sexually selected secondary characteristics.

**Figure 4.** Insulin signaling pathway (after Eijkelenboom & Burgering, 2013). When insulin receptors (InR; blue oval in the diagram) are activated through the nutrition-dependent release of insulin-like peptides or insulin-like growth factors, AKT gets activated through the activation of phosphoinositide 3-kinase or PI3K. This leads to a downregulation of FoxO (Forkhead Box, subgroup O), which inhibits cell proliferation. For more details, see text and Eijkelenboom & Burgering (2013).
such questions.) For this case study, we presented the students with work by Douglas Emlen and colleagues, who used the large, charismatic horned beetle Trypoxylus dichotomus to assess the importance of insulin receptors in the evolution of allometric relationships (Emlen et al., 2012). The researchers conducted an RNAi experiment that targeted insulin receptors in last-instar larvae and measured the resulting adult length of three traits that differed in their allometric relationships (aedeagus, wing, and head horn). We introduced our students to this study by presenting them with length measurements of the three traits of control animals, which were plotted against body size (available in Emlen et al., 2012). Using these data, the students were asked the following questions:

(a) Which of the three types of allometric relationships apply to each trait in untreated individuals (isometric, hypometric, hyperallometric)?

(b) Which of the three traits is most/least sensitive to nutrient availability?

(c) Which of the three traits has the highest/lowest number of insulin receptors under normal conditions?

(d) How would you expect RNAi of the insulin receptor to affect each of the traits?

(e) Do the results of the RNAi experiment match your predictions?

After team discussions, most students correctly predicted that horns, which are a hyperallomeric trait, are expected to be most sensitive to nutrient availability (as they grow faster than body size). Based on this answer, pupal horn tissues are expected to bear a large number of insulin receptors on the membrane of each cell. Consequently, horns are also expected to be particularly sensitive to a reduction of insulin receptors via RNAi and, thus, are expected to experience the most significant growth reduction for a given body size. On the other hand, aedeagi exhibit a hypometric relationship and, thus, are expected to be less sensitive to nutrient availability, which might be mediated by a reduced number of available insulin receptors on the cell membrane. Given their limited response to nutrient availability, a further reduction in insulin receptors is expected to lead to little change in the overall growth rate of aedeagi, which is supported by the researchers’ results.

● Conclusion

Insects, with their relatively simple yet extraordinarily varied body plans, were particularly integral as research subjects in the advent and subsequent flourishing of evo-devo as a discipline. In turn, our understanding of insect developmental genetics and morphological evolution has grown disproportionately in relation to other taxonomic groups. Here, we have argued that a more comprehensive assimilation of evo-devo research into entomology instruction might enhance and solidify students’ understanding of insect biology beyond that achieved through traditional approaches.

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References


