Chapter 5
Theme and Variation in the Development of Insect Mouthparts

David R. Angelini and Frank W. Smith

Abstract Insect mouthparts are serially homologous appendages. As such, their development and evolution are nonindependent. Arthropod appendages share similarities in their developmental origins and underlying genetics. Here, we review the development, specification, and patterning of insect mouthparts, with comparisons to the legs of *Drosophila melanogaster*. The expression and function of genes in the arthropod head give clues as to the homology of the labrum. The activity of Hox genes establishes appendage-specific gene expression and interactions allowing for the development of unique appendage types. Many similarities exist in the patterning of gnathal appendages and legs; however, unique variations in gene function in each appendage type provide clues to the developmental origins of mouthpart morphologies. We examine what is known about mouthpart patterning in mandibulates, as exemplified from several beetle species, as well as in the proboscis of *Drosophila melanogaster* and in the hemipteran rostrum of *Oncopeltus fasciatus*. With these findings in mind, we reflect on the evolution of serially homologous structures.

5.1 Introduction

The mouthparts and other appendages of arthropods possess a versatile developmental program. The segmented body plan of these animals makes it possible for the redeployment of a conserved developmental system, which nevertheless admits variations enabling evolution and adaptation. Arthropods confront their environment with a varied array of tools for different lifestyles. Their success seems supreme in species diversity, if not also anatomical disparity. As far as we now understand it, this diversity arises from a shared set of developmental events and the genes that control them. Nevertheless, investigations of comparative developmental biology

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and genetics have uncovered a mixture of conservation and divergence in insect appendage development.

Here, we will attempt to contextualize the patterns of the evolution in insect mouthpart and appendage development through analogy to the musical ideas of "theme and variation." Compared between species and between appendage types, mouthparts and other insect appendages are both special and serial homologs, respectively. These appendages share a great deal in their developmental origins and underlying genetics. This is the common "theme." But key differences exist and influence the generation of morphological variations. While consistent themes run throughout, individual variations enable novel life histories.

In this chapter, we will review thematic aspects of development common to arthropod species and appendage types, reflecting primarily on the mouthparts of insects. We will also explore variations that allow for unique appendage types and for the unique features of individual lineages.

Theme and Variation In classical western music, the compositional technique of theme and variations uses a theme as the central musical idea of the piece, usually a memorable melody or chord progression. As the piece progresses, the theme is repeated again and varied in a different way. This cycle continues several times, providing the structure for the piece of music. Often the conclusion returns more closely to the theme or has a dramatic or poignant variation.

5.2 Homology: Theme and Variation

The shared developmental features of insect appendages reflect their complex evolutionary history, and it is useful to distinguish between the different ways in which these structures are related to one another. An important issue is that morphology and developmental similarities reflect both a history of common descent (homology or, formally speaking, special homology) and the shared deployment of developmental programs at different positions in the body (serial homology).

The first appreciation of morphological similarity in western science was closer to our current notion of serial homology and explicitly implicated development. The poet, statesman, and botanist, Johann Wolfgang von Goethe, carefully observed the development of plants and noted the similarities between leaves and floral organs (1790). Goethe described that these different structures grew from a similar meristem but diverged as development proceeded. He described the differences in their structures as arising from differences in "expansion" or "contraction" (Pfau 2010), although it seems clear he meant more than simply allometric differences. Goethe's observation of this connection has direct historical continuity to our present idea of serial homology. Moreover, Goethe also contemplated the implications of his idea for species diversity. He considered that his model of development could, starting from the "Urpflanze" (the archetypal or primordial plant), "invent plants without limit." This concept could also be universal: "The same law will permit itself to be applied to everything that is living" (Goethe 1814; Pfau 2010).

It is perhaps ironic that the term "homology" was coined by Richard Owen (1843), who vocally opposed the idea of species evolution. Nevertheless, Owen clarified the ideas first expressed by Goethe, crediting him for his influential observations (1848). Owen explicitly defined what he called "serial homology" as the repeated appearance of structures, such as vertebrae, within the body of an animal. He distinguished this from "special homology," which he described as "correspondency of a part or organ, determined by its relative position and connections, with a part or organ in a different animal" (1848). Without recognizing the possibility of evolution, Owen drew the distinction to what he called "general homology," "... that in which a part or series of parts stands to the fundamental or general type, and its enunciation involves and implies a knowledge of the type on which a natural group of animals ... is constructed."

After Darwin, the concepts of special and general homology collapsed into one, as writers on the subject came to understand (special) homology as arising from shared ancestry. By the mid-twentieth century, Boyden (1943, 1947) argued that the literature had gone too far and confused serial and special homology, complicating the use of characters in taxonomy. In the 1980s, evolutionary biologists considering the implications of development (e.g., Van Valen 1982; Roth 1984) and developmental biologists considering the implications of evolution (e.g., Raff and Kaufman 1983; Wagner 1989) began to reconsider concepts of homology, arguing for a more mechanistic basis and drawing clear distinctions between special and serial homology.

In recent decades, detailed mechanistic studies of development in anatomically disparate organisms (e.g., Hinman et al. 2003; Davidson 2006) have meant that considerations of the evolution of characters often depend on consideration of their generative mechanisms. Günter Wagner (2007) has argued that the unit of homology should be considered to be the developmental genetic system responsible for the identity of a particular trait, what he terms the character identity network (ChIN).

We will return to the idea of homology in our conclusions and explore how insect appendage development reflects general principles in the evolution of homologous structures. The anatomy of insect mouthparts will be detailed elsewhere in this volume. So we will only briefly summarize their structure here, focusing on taxa relevant to studies of development.

5.3 Overview of Insect Mouthpart Anatomy

The ancestral and most common state of insect mouthparts is the mandibulate type (Grimaldi and Engel 2005; Misof et al. 2014), which is fixed in several prominent orders such as Odonata, Orthoptera, Coleoptera, and Hymenoptera (Marshall 2006). Mandibulate mouthparts are primarily used for chewing, and they appear in both generalist and specialist taxa. From anterior to posterior, the mouthpart appendages consist of the labrum, mandibles, maxillae, and labium (Snodgrass 1930, 1935). The labrum's status as an appendage remains controversial (e.g., Popadić et al. 1998; Haas et al. 2001; Kimm and Prpic 2006; Posnien et al. 2009), and this question is considered below. Anatomically, the labrum acts as an upper lip and roof to the oral cavity. The mandibles are unjointed appendages used in chewing, and they are typically robust and well-muscled. The maxillae are paired, jointed appendages, which branch distally. The basal-most segment of the maxilla, the cardo, is jointed to the ventral head. The next segment is the stipes, which articulates with two medial endites, the lacinia and galea, which are fringed with setae in many species. Laterally, the stipes is also jointed to the maxillary palps. The palps typically consist of multiple segments, although their number may vary between different taxa. The palps typically function in the recognition of food. Chemosensory receptors on the surface of the palps aid the insect in identifying its target food (Snodgrass 1930; Chapman 1998). The posterior mouthpart appendage is the labium. The proximal labial segments fuse medially, forming the mentum and prementum. These segments may be jointed, or the joint between them may fuse, as in *Tribolium* (Sokoloff 1972; Angelini et al. 2012a). Medially, the prementum articulates to a set of endites in most species. There may be as many as four labial endites, two medial glossae and two lateral paraglossae, although these are reduced or fused in some taxa (Snodgrass 1930, 1935). Lateral of the endites, the labium also articulates with a pair of palps, similar in their structure and function to the maxillary palps. The number of labial palpomeres also varies among taxa. The hypopharynx is a fleshy, non-appendicular structure that acts as a tongue or the bottom of the oral cavity in some taxa. While not prominent in many mandibulate insects, the hypopharynx is an essential component of some derived mouthpart morphologies.

Fossils and phylogenetic evidence establish mandibulate anatomy as the ancestral state for insects (Grimaldi and Engel 2005; Misof et al. 2014). Among extant orders, at least 24 of the 32 (as recognized by Misof et al. 2014) are characterized by mandibulate mouthparts. The development of mandibulate mouthparts has been examined in model species representing multiple orders, including the cricket *Gryllus bimaculatus* (reviewed by Liu and Popadić 2017) and the beetle species *Tribolium castaneum* (Angelini et al. 2012a), *Onthophagus taurus* (Simonnet and Moczek 2011), and *Cyclommatus metallifer* (Gotoh et al. 2017).

However, some of the most successful groups of insects have exploited variations on the mandibulate theme. Among these novel morphologies is the principle insect model of development and genetics, the fruit fly *Drosophila melanogaster*. Diptera are characterized by the modification of mouthparts to piercing or sponging functions. In Muscomorpha, such as *D. melanogaster*, this involves the reduction and fusion of mouthpart appendages and surrounding head structures into a proboscis. The labial palps are absent, and the labium ends in a modified area called the labellum that is used for collection of liquid or particulate food (Snodgrass 1944). Mosquitos have evolved bladelike mandibles and maxillary laciniae, with an elongated hypopharynx used to secrete saliva (Snodgrass 1959). Emerging models of vector biology, such as *Anopheles gambiae* (Adolfi and Lycett 2018), have the potential to serve as comparative models of mosquito mouthpart development in the future.

The milkweed bug *Oncopeltus fasciatus* has also emerged as an informative system for developmental genetics (Chipman 2017; Panfilio et al. 2018), and this species represents the diverse Hemiptera. In this order, the labium is modified into a medially fused rostrum with multiple joints and no endites, while the mandibles and maxillae form thin stylets used in piercing and fluid feeding. Lepidoptera are another lineage in which existing model species, such as the silk moth *Bombyx mori* (Tomita and Kikuchi 2009; Ando et al. 2018), may be amenable to developmental genetic studies of mouthparts. Lepidopteran larvae retain chewing mandibulate mouthparts. Except for the early-branching lineage of Micropterigidae, adult Lepidoptera have evolved mouthparts in which the maxillary galeae form a proboscis typically used for nectar feeding (Krenn 2010). Secondarily, adults of the ghost moths (Hepialoidea) have reduced or absent maxillary palps and galeae. The mouthparts of these moths are vestigial, and the adults do not feed (Powell and Opler 2009). A fascinating novelty exists in Prodoxidae, where female Yucca moths develop an enlarged maxillary palpomere that is used independently of the proboscis to pollinate their host plant (Davis 1967; Pellmyr and Krenn 2002).

Other groups present intriguing mouthpart modifications, but few models currently lend themselves to developmental genetic investigations. For example, Thysanoptera present an interest comparison to Hemiptera, their sister taxon. The mouthparts of thrips are asymmetrical, with a single left mandible modified to form a piercing stylet. The maxillae differ in size, but each possesses a medial stylet and a small lateral palp. The thysanopteran labium is much closer in morphology to that of mandibulates. It is symmetrical, with a medial mentum and prementum, ending distally in medial endites and lateral palps (Jones 1954; Hunter and Ullman 1992). Siphonaptera (fleas) are another insect group with independently derived piercing mouthpart morphologies (Snodgrass 1946). In fleas the mandibles are absent, but bladelike mouthparts are formed by elongation of the labrum and laciniae. The maxillae and labium retain palps. Unfortunately, despite their medical importance, developmental studies of Siphonaptera have lagged behind other groups.

5.4 Development of Insect Mouthparts

5.4.1 The Embryonic Origins of Insect Mouthparts

In all hemimetabolous and many holometabolous insects, the mouthparts originate as ventral-lateral outgrowths from the embryo (Fig. 5.1; Snodgrass 1928; Butt 1949; Van Horn 1966). Limb buds appear soon after segment formation. Therefore, in species with short germ band development, the limb buds of the gnathal and thoracic segments appear before obvious external segmentation in the abdomen is completed. Initially, limb buds consist exclusively of ectoderm, but mesodermal cells from the body of each segment contribute to the appendages forming the muscles (Eastham 1931;

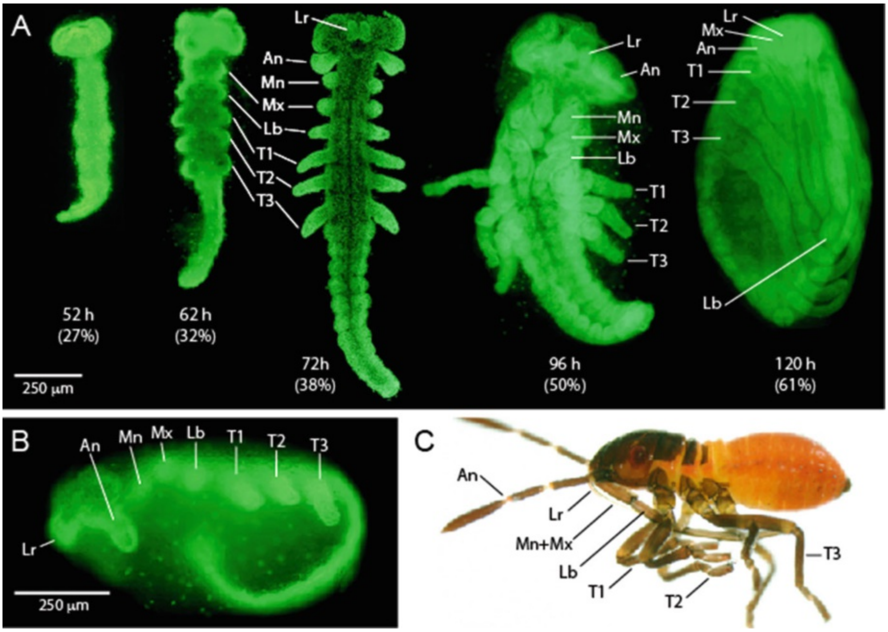


Fig. 5.1 In most insect species, appendages develop from three-dimensional embryonic limb buds, such as in the milkweed bug *Oncopeltus fasciatus*. (**a**) *O. fasciatus* embryos of different ages are shown stained with Sytox, a fluorescent dye that binds to DNA, highlighting nuclei. Ages are given as hours post egg-laying and as a percentage of total average developmental time. Embryos have been dissected away from yolk for clarity. (**b**) A 72-h embryo stained with Sytox is shown from a lateral view with the yolk intact. (**c**) Lateral view of an *O. fasciatus* first-instar nymph. Notice that appendages are visible early, before abdominal segmentation is complete. The limb buds grow rapidly, and by 96 h, regionalization within the appendages is apparent. The labial appendages are initially separate but migrate ventrally, and by 120 h, they fuse together at the midline. *An* antenna, *Lr* labrum, *Mn* mandible, *Mx* maxilla, *Lb* labium, *T1–3* thoracic legs

Heming 1980). In Holometabola, muscle stem cells are associated with the imaginal discs and also give rise to the appendicular muscles at metamorphosis (Snodgrass 1935). During the germ band stage, specific gene expression establishes the components of differing character identity networks to define each appendage type.

5.4.2 *Postembryonic Development of Appendages*

Ancestrally, insects have a more-or-less direct development of the body plan. While adult structures such as wings and genitalia only appear after the adult molt (or in the subimago of Ephemeroptera; Edmunds and McCafferty 1988), among members of the hemimetabolous orders, which lack a complete metamorphosis, juveniles hatch with appendages similar in structure to those of the adult, differing only in relative size and cuticle or sensory features. Nevertheless, the number of segments in some distal appendage structures can vary by developmental stage. For example, in *Oncopeltus* juveniles, the legs have two tarsomeres on each leg, while adults have three, apparently due to the formation of a novel joint within the distitarsus.

In the Holometabola, species undergo a complete metamorphosis with a non-motile pupa. During this stage, appendages undergo a more dramatic repatterning. In most holometabolous orders, legs and mouthparts are present in juveniles but have a less complex morphology compared to adults. For example, the distal segments of *Tribolium* juvenile legs are much smaller than in the adult, and the tibiotarsus exists as a single segment that will become two in the adult (Angelini et al. 2012b). Adult structures are produced by cells from corresponding larval structures (Švácha 1992). An extreme "indirect" form appendage development exists in some Holometabola. *Drosophila* is a familiar example, in which larval appendages are visible externally only as small sensory Keilin's organs (Dambly-Chaudière and Ghysen 1986). In fruit flies and other Muscomorpha, most of the larval epidermis is polyploid (Smith and Orr-Weaver 1991) and must be replaced during metamorphosis. Imaginal discs give rise to the appendages and much of the surrounding body wall, while imaginal histoblasts produce to the remainder of the adult cuticle (Mandaravally Madhavan and Schneiderman 1977).

5.5 The Mystery of the Labrum

The labrum is an apical appendage-like structure on the insect head. It functions as the upper lip of insects (Snodgrass 1935); houses many sensory structures, such as setae, pressure receptors, trichoid sensilla, and coeloconic sensilla (Smith et al. 2014b); and serves a chemosensory function (Ortega-Hernández and Budd 2016). Several long-standing questions regarding the labrum have perplexed biologists (Budd 2002; Scholtz and Edgecombe 2006; Ortega-Hernández et al. 2017). Is the labrum a segmental structure, and if so, which segment is the labrum associated with? Is the labrum homologous to the paired ventral appendages that characterize insects and other arthropods? Lastly, what structure, if any, is the labrum homologous to in most distant relatives of Arthropoda? Several hypotheses have been proposed for each of these questions based on comparative studies of morphology and embryogenesis (Fig. 5.2). More recently, advances in developmental genetic techniques have provided an additional approach to testing hypotheses regarding the nature of the labrum. Here, we review the hypotheses for the nature of the insect labrum and summarize recent advances in our understanding of the labrum based on studies of developmental genes.

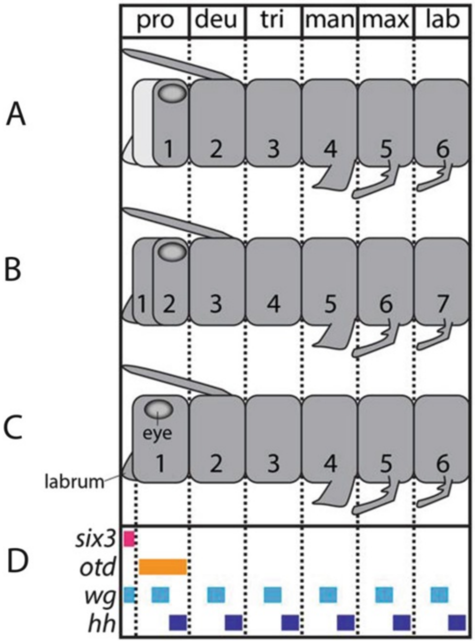


Fig. 5.2 Gene expression and models of labrum identity in the insect head. Segments are numbered according to the different models. See main text for references. Segmental regions are shaded dark gray, and non-segmental regions are shaded light gray. (**a**) The protocerebral region is composed of a segmental and non-segmental region. (**b**) The protocerebral region is composed of two segments. (**c**) The protocerebral region is composed of a single segment and does not include non-segmental tissue. (**d**) Developmental gene expression patterns. The boundaries between *wg* and *hh* expression mark the parasegmental boundaries. The segment polarity gene *wg* is expressed in the labrum and in the ocular region of the developing protocerebrum. *deu* deutocerebral segment, *lab* labial segment, *man* mandibular segment, *max* maxillary segment, *pro* protocerebral region, *tri* tritocerebral segment

5.5.1 Where Is the Axial Origin of the Labrum?

The labrum has been hypothesized to be a component of the intercalary segment—the segment that gives rise to the tritocerebral brain neuromere (Butt 1960; Haas et al. 2001), the acron—an unsegmental anterior-most region of the insect head (Brusca and Brusca 2003), or the first segment of the insect head (Budd 2002). The intercalary segment hypothesis is supported by several pieces of evidence, each of which has recently come under scrutiny in the literature. The first piece of evidence is based on the position of the labrum in the insect head. The stomodeum, which the labrum is closely associated with, sits somewhere between the intercalary segment and the antennal segment in models of insect head segmentation (Rempel 1975; Schmidt-Ott and Technau 1992; Rogers and Kaufman 1997; Haas et al. 2001). However, during embryogenesis, the stomodeum migrates posteriorly from an apical-most region (Khila and Grbić 2007). Furthermore, expression of the gene *six3*, which marks the apical-most region of the developing body axis of annelids, hemichordates, and onychophorans, also marks the labrum of insects (Fig. 5.2d; Steinmetz et al. 2010). These developmental studies suggest that the labrum originates in an apical position in the insect body axis, rather than in the intercalary segment, i.e., the ultimate position of the labrum does not reflect the position at which the labrum originates during embryogenesis. The second piece of evidence favoring an intercalary segment origin for the labrum is the fact that the labrum is innervated by the tritocerebral brain neuromere in the locusts *Schistocerca gregaria* and *Locusta migratoria* (Boyan et al. 2002). However, the labrum is innervated by the deutocerebrum in the horseshoe crab *Limulus polyphemus* (Mittmann and Scholtz 2003). The innervation of the labrum by either the deutocerebrum or tritocerebrum in euarthropods may represent derived conditions related to the ultimate position of the labrum, rather than its segmental origin (Scholtz and Edgecombe 2006; Bitsch and Bitsch 2010). Third, in the crustacean *Porcellio scaber* (Abzhanov and Kaufman 1999) and the centipede *Lithobius atkinsoni* (Hughes and Kaufman 2002b), the Hox gene *labial* (*lab*), which labels the intercalary/tritocerebral segment in all arthropods, is also expressed in the labrum (Haas et al. 2001). However, *lab* is not expressed in the labrum of other euarthropods investigated, including insects (Mlodzik et al. 1988; Rogers and Kaufman 1997; Peterson et al. 1999; Nie et al. 2001; Posnien and Bucher 2010), chelicerates (Damen et al. 1998; Sharma et al. 2012), and millipedes (Janssen and Damen 2006). This more comprehensive survey of *lab* expression suggests that its expression in the labrum of *P. scaber* and *L. atkinsoni* is likely a derived condition of the lineages leading to these species and is not indicative of the segmental origin of the labrum. In summary, most researchers now agree that the labrum originates in the insect protocerebral region. This hypothesis is supported by the expression of *six3* in the labrum (Fig. 5.2d; Steinmetz et al. 2010) and the fact that the labrum migrates posteriorly from an apical-most position during insect development (Khila and Grbić 2007).

5.5.2 Is the Labrum a Segmental Structure?

While a consensus exists regarding the position of the labrum on the protocerebrum, there remains debate regarding the segmental nature of the protocerebrum. Current debates revolve around whether the protocerebrum represents a single segment, two fused segments, or a composite between a non-segmental and a segmental region. These debates have important implications for interpretations of the evolution of the labrum.

The existence of a non-segmental apical region in the insect head, and the heads of other euarthropods, originated with the Articulata hypothesis, which posits a sister-group relationship between Euarthropoda and Annelida, and a common origin of segmentation between these lineages (Scholtz 2002; Scholtz and Edgecombe 2006). The apical-most region of Annelida, referred to as the prostomium, lacks signatures of segmentation that are exhibited by body segments, such as nephridia and coelomic sacs, and unlike the body segments of Clitellata (earthworms and leaches), it does not develop from a posterior growth zone (Nielsen 2001; Ackermann et al. 2005; Scholtz and Edgecombe 2006). In polychaetes, distinct morphogenetic mechanisms underlie larval and juvenile segment development, but neither of these mechanisms is involved in development of the larval episphere, which gives rise to the prostomium (Ackermann et al. 2005; Scholtz and Edgecombe 2006). Therefore, this anterior-most region of the body axis may truly be regarded as non-segmental in nature (Scholtz and Edgecombe 2006). By extension, if segmentation is homologous between annelids and arthropods, then arthropods should exhibit an anterior-most non-segmental region.

In annelids, the prostomium is marked by *six3* expression during development, while the first segment—the peristomium—is marked by expression of the insect homolog of the gene *orthodenticle (otx)*. Likewise, *six3* marks the anterior-most region of the body axis of insects and other arthropods, while *otx* marks a slightly more posterior region (Fig. 5.2d; Steinmetz et al. 2010). These expression domains both lie within the protocerebral region in insects and other arthropods. Therefore, in accordance with the Articulata hypothesis, the protocerebral region would represent a composite between an anterior non-segmental region and a posterior segmental region (Fig. 5.2a), much as the annelid head is composed of the prostomium and the peristomium. The labrum lies within the expression domain of *six3*, in insects and other euarthropods (Steinmetz et al. 2010). Since this region is predicted to be homologous to the annelid prostomium—a non-segmental region, according to the Articulata hypothesis, the labrum would represent a non-segmental structure (Fig. 5.2a).

Molecular analyses have revealed that arthropods and annelids are not closely related (Aguinaldo et al. 1997; Dunn et al. 2008). Based on these analyses, the Articulata hypothesis has been replaced by the Ecdysozoa hypothesis, which posits that insects and other arthropods are more closely related to several unsegmented phyla than they are to annelids. The Ecdysozoa hypothesis suggests that segmentation evolved independently in Euarthropoda and Annelida. While the Ecdysozoa hypothesis has now reached a consensus in the field (Giribet and Edgecombe 2017), whether an apical unsegmented region exists in the head of insects and other euarthropods remains an open question (Budd 2002; Scholtz and Edgecombe 2006; Posnien et al. 2010). This possibility might be expected, if annelids and euarthropods evolved segmentation in parallel from shared ancestral developmental mechanisms that were reiterated along an unsegmented body axis, as has been proposed (Chipman 2010). Two observations based on studies of the red flour beetle *Tribolium castaneum* suggest that an apical non-segmental region does exist within the protocerebrum of insects (Posnien et al. 2010). First, the V-shaped median apical-most region that gives rise to the labrum lacks the parasegment-like gene expression patterns that reliably demarcate body segments along the rest of the insect body axis (Fig. 5.2d; Posnien et al. 2009, 2010). Second, the gene regulatory network that patterns the V-shaped region is not reiterated in segmental patterns (Li et al. 1996; Schroder et al. 2000; Economou and Telford 2009; Posnien et al. 2009; Steinmetz et al. 2010). Taken together, these observations suggest that the insect protocerebrum may be composed of a median apical non-segmental region *and* a posterolateral segmental region.

The remaining hypotheses regarding the segmental nature of the protocerebrum region agree that this region is segmental. By extension, these hypotheses argue that the labrum is a segmental structure. However, they disagree about the number of segments that compose the protocerebrum. In one hypothesis, the protocerebrum is composed of a fusion between two ancestrally independent segments (Fig. 5.2b; Strausfeld 2012; Cong et al. 2014). In insects and other arthropods, two regions can be recognized within the protocerebrum—the anterior region is referred to as the prosocerebrum and includes the labrum and the posterior region is referred to as the archicerebrum and includes the optic lobes and mushroom bodies of the brain (Urbach and Technau 2003). According to this hypothesis, the labrum represents a fused pair of segmental appendages of a protocerebral segment, while the stalked eyes of stem group euarthropods—homologs of insect compound eyes—represent the segmental appendages of an archicerebrum segment (Strausfeld 2012; Cong et al. 2014). In both insects and other euarthropods, segment polarity genes are typically expressed in a one-stripe per segment pattern but are expressed independently in both the labrum and ocular regions of the protocerebrum (Fig. 5.2d; Damen 2002; Farzana and Brown 2008; Posnien et al. 2009; Janssen 2012), which lends some developmental support to this hypothesis (Ortega-Hernández et al. 2017).

The lack of fossil evidence for the transition between a leg and a stalked eye, a prediction of the dual segment origin of the protocerebrum, challenges this hypothesis (Ortega-Hernández et al. 2017). Additionally, it now seems clear that the insect protocerebral region is homologous to the head of tardigrades (Smith et al. 2016, 2018) and the eye-bearing segment of onychophorans (Eriksson et al. 2010). Stalked eyes evolved in the euarthropod lineage, after this lineage diverged from Tardigrada and Onychophora (Park et al. 2018). Therefore, the dual segment origin predicts that two appendage pairs should be found in the protocerebral region of tardigrades and onychophorans, but a single appendage pair—the frontal appendages—is found in this region in onychophorans, and either no appendages or a single appendage pair is found in this region in tardigrades, depending on whether the teeth-like stylets of tardigrades are derived from legs or not (Nielsen 2001).

The remaining hypothesis argues that the protocerebrum represents a single segment, with the labrum representing a fused appendage pair of this segment (Budd 2002; Budd and Telford 2009; Ortega-Hernández et al. 2017). According to this hypothesis, the independent expression domains of segment polarity genes in the insect protocerebrum are the result of co-option of these genes for novel functions in the protocerebrum, possibly in development of the ocular lobes (Ortega-Hernández et al. 2017). In this hypothesis, each segment of ancient panarthropods housed a pair of appendages, and the labrum represents the appendage pair of a single protocerebral segment (Budd 2002; Budd and Telford 2009; Ortega-Hernández and Budd 2016). This hypothesis aligns well with recent conclusions about the homology of the protocerebral region across Panarthropoda based on developmental studies (Smith et al. 2016, 2018) and fossil evidence (Park et al. 2018). Yet it remains possible that the protocerebrum evolved from a fusion of two segments. If so, based on current evidence, this fusion must have happened in the stem group of Panarthropoda (Ortega-Hernández et al. 2017), rather than in the stem group of Euarthropoda (Strausfeld 2012; Cong et al. 2014).

5.5.3 Is the Labrum Serially Homologous to the Ventral Appendages?

Studies of labrum development have clear consequences for our interpretations of the homology of this structure to the ventral appendages of insects and other euarthropods—including the gnathal appendages. One way to gauge homology is to test whether similar mechanisms control the development of the labrum and the ventral appendages. Like the ventral appendages, the labrum originates as paired bud-like structures during insect development (Scholtz and Edgecombe 2006; Posnien et al. 2009). Furthermore, the distal appendage-patterning gene *Distal-less* (*Dll*) and other components of the appendage-patterning network are active in the developing labrum of several insect species investigated (Angelini and Kaufman 2004; Ronco et al. 2008; Ohde et al. 2009; Posnien et al. 2009; Simonnet and Moczek 2011; Smith et al. 2014b; Yoshiyama et al. 2013). These results support homology between the labrum and the ventral appendages.

Although similar mechanisms control development of the labrum and the ventral appendages, there are compelling differences. The ventral appendages develop at parasegmental boundaries. The Wnt signaling protein encoded by *wingless* (*wg*) is expressed on the anterior side of parasegmental boundaries, and *hedgehog* (*hh*) is expressed on the posterior side of the boundaries (Fig. 5.2d; Hidalgo 1991; Posnien et al. 2009). In *T. castaneum* and other insects, *wg* and *hh* are required for activation of *Dll* (Morata 2001; Posnien et al. 2009). Targeting *hh* or *wg* with RNAi during *T. castaneum* embryogenesis leads to loss of *Dll* expression where ventral appendages normally develop and, in the case of *hh*, complete deletion of all ventral appendages (Posnien et al. 2009). By contrast, the labrum does not develop at a parasegmental boundary, and RNAi targeting *hh* or *wg* treatments does not affect *Dll* expression in the labrum or lead to deletions of the labrum (Posnien et al. 2009). These results suggest that there are no parasegmental boundaries in the region where the labrum develops and that different mechanisms activate *Dll* expression in the labrum compared to ventral appendages. These conclusions are consistent with the hypothesis that the labrum develops in a non-segmental region of the insect head and suggest that the labrum is not a serial homolog of the ventral appendages (Posnien et al. 2009). Additionally, the Notch pathway activates *Dll* expression in the labrum, but not in the ventral appendages (Siemanowski et al. 2015).

If the labrum is not a serial homolog of the ventral appendages, then why are there are so many similarities between labrum development and ventral appendage development? One hypothesis is that the labrum is a novel structure that evolved by co-option of the ventral appendage-patterning network (Posnien et al. 2009;

Simonnet and Moczek 2011; Smith et al. 2014b). This hypothesis underpins a counterintuitive possibility. As an appendage, the labrum may not be homologous to the ventral appendages, while the developmental mechanisms that control development of the labrum and ventral appendages may be homologous.

5.5.4 *How Does the Insect Labrum Relate to Structures in Other Animals?*

A protocerebral appendage pair is predicted to be an ancient characteristic of Panarthropoda (Budd 2002). This ancestral appendage pair is thought to have given rise to the frontal appendages of onychophorans and possibly the teeth-like stylets of tardigrades (Nielsen 2001). This ancient appendage pair is exemplified by the "great appendages" of stem group euarthropods (Budd 2002). According to this hypothesis, the insect labrum—and the labra of other euarthropods—evolved from this ancient appendage pair. This hypothesis finds developmental support from expression of *six3*; *six3* is expressed in the developing antenna-like frontal appendages of onychophorans and the euarthropod labrum (Steinmetz et al. 2010; Eriksson et al. 2013). More recently, several genes that are expressed in the developing euarthropod labrum were found not to be expressed in the developing onychophoran frontal appendage, casting doubt on the significance of expression patterns of a single gene, *six3*, for inferring homology of the euarthropod labrum and onychophoran frontal appendage (Janssen 2017b). In other words, the fact that *six3* is expressed in both the labrum and frontal appendages may reflect the fact that they both develop in a homologous region of the body axis, rather than representing evidence that they share structural homology. On the other hand, differences in developmental patterning mechanisms should not be surprising, given how morphologically different the euarthropod labrum is compared to the onychophoran frontal appendages. Additional studies of labrum development and frontal appendage development need to be performed to better gauge the homology of these structures.

5.5.5 *Current Outlook on Identity and Evolution of the Labrum*

Although there is much to be determined regarding the origin of the labrum, the above discussion reveals three elements related to the evolution of the labrum that have reached a near consensus among zoologists. First, fossil evidence (Cong et al. 2014; Park et al. 2018) and developmental studies of Onychophora (Eriksson et al. 2010, 2013) strongly support a model in which an ancient ancestor of euarthropods had an appendage pair on the protocerebral region. Second, the labrum develops in the protocerebral region of the body axis (Steinmetz et al. 2010). Third, similar mechanisms control patterning of both the labrum and the ventral appendages (Smith et al. 2014b). However, determining whether the labrum is homologous to frontal appendages of onychophorans and ancient panarthropods and whether it is homologous to the ventral appendages requires additional studies. An important step toward addressing these questions will be to determine the segmental composition of the protocerebral region. New paleontological insights and developmental studies of a more diverse group of insects, additional euarthropods, and even onychophorans and tardigrades may be required to finally solve the mystery of the labrum.

5.6 Identity Specification of the Gnathal Appendages

The body plans of animals are established early in embryonic development. Anterior-to-posterior axial gradients activate a series of conserved transcription factors in adjacent and sometimes overlapping domains. Loss of function in these genes results in homeosis, the development of one anatomical structure in the position normally held by another. In many species these genes are linked in adjacent positions on the chromosomes. Their homeotic mutant phenotypes and linkage in a genetic complex gave them their name: Hox genes. In the 1990s and 2000s, evolutionary developmental biology (evo-devo) grew as a field in part by exploring the connections between Hox gene function and arthropod body plan variations (reviewed by Hughes and Kaufman 2002b; Angelini and Kaufman 2005). These genes are active during embryonic development, but the specification of appendage identity is an ongoing process, as evidenced by the transformation of appendages during metamorphic or juvenile-to-adult development following knockdown by RNA interference (e.g., Tomoyasu et al. 2005; Wasik et al. 2010; Aspiras et al. 2011).

5.6.1 The Mandible

The mandible is the anterior-most head appendage that is not associated with a brain-housing segment. This appendage articulates with the head capsule but otherwise lacks joints. In zoological terms, it consists of the coxopodite (proximal) component, but not the telopodite (distal) component of the generalized insect appendicular appendage (Snodgrass 1935). In line with its coxopodite identity, the insect mandible lacks expression of the telopodite maker *Distal-less* during embryogenesis (Rogers et al. 2002). Additional gene expression studies suggest that the mandible is primarily composed of single endite of a single basal podomeres (Coulcher and Telford 2013).

While genetic screens of *Drosophila melanogaster* have laid the foundation for our understanding of how appendage identities are specified during development, fruit flies lack mandibles. For this appendage type, knowledge of mandible identity specification arose from studies of other insect species. As with other gnathal

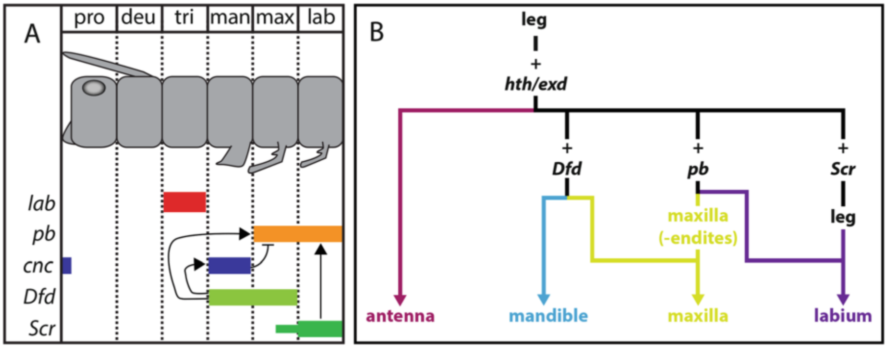


Fig. 5.3 Head appendage identity specification based on studies of *T. castaneum* and other insects. See main text for references. (**a**) Expression domains of the Hox genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), and *Sex combs reduced* (*Scr*) and the gene *cap'n'collar* (*cnc*) in the insect head. Known regulatory interactions are shown. Arrows indicate activation of expression. The horizontal bar indicates repression of expression. The thin line indicates a more restricted expression domain of *Scr* in the maxillary segment. (**b**) A model for appendage identity specification in insects. The default identity is leg (top). Expression of appendage identity selector genes in appendage anlagen (+ gene name) modifies the default leg state. Pathways leading to modified appendage identities are color-coded. *deu* deutocerebral segment, *lab* labial segment, *man* mandibular segment, *max* maxillary segment, *pro* protocerebral region, *tri* tritocerebral segment

appendages, the Hox genes play important roles in regulating mandible identity. In winged insects, the only Hox gene that is strongly expressed in the mandible is *Deformed* (*Dfd*) (Fig. 5.3a; Rogers and Kaufman 1997; Brown et al. 1999a; Hughes and Kaufman 2000; Rogers et al. 2002; Angelini et al. 2005). However, the insect ortholog of *Hox3*, *zerknüllt* (*zen*), which is typically expressed extraembryonically during insect development (Schmidt-Ott et al. 2010), is also expressed in a more typical Hox gene pattern in the apterygote insect *Thermobia domestica* (Hughes et al. 2004). In this species, *Hox3* is expressed in the mesoderm of the developing mandibles and maxillae. *Hox3* was most likely also expressed in the developing mandibles of the last common ancestor of insects, given that it is expressed in the mandibles of crustaceans (Papillon and Telford 2007) and centipedes (Hughes and Kaufman 2002a) and given that Zygentoma—the apterygote lineage that includes *T. domestica*—is an out-group of all winged insects that have been investigated (Yeates et al. 2016). Like in *T. domestica*, *Hox3* expression is restricted to the mesodermal layer of the developing mandibles of the crustacean *Daphnia pulex* (Papillon and Telford 2007), suggesting that this gene played a role in regulating development of mesodermal derivatives in the mandibles ancestrally in insects. Additionally, *Sex combs reduced* (*Scr*) is expressed at low levels in the mandibles of *T. domestica* (Passalacqua et al. 2010).

At this juncture, the function of *zen* and *Scr* in the developing mandibles of *T. domestica* is unknown. However, the function of *Dfd* during mandible development has been investigated in insects with generalized mandibulate mouthparts—the flour beetle *Tribolium castaneum*—and insects with highly derived mouthparts—the milkweed bug *Oncopeltus fasciatus*. In *T. castaneum*, null *Dfd* mutants and RNA interference (RNAi) targeting *Dfd* result in nearly complete transformations of the larval mandible to antenna (Fig. 5.3a; Brown et al. 1999b). In this species, Dfd activates the transcription factor-coding genes *cap'n'collar* (*cnc*) and *paired* (*prd*) during embryogenesis (Coulcher and Telford 2012). Dfd activates expression of *cnc* broadly across the mandible segment, including in the developing mandibles, and *prd* specifically in the endites of the mandibles (Fig. 5.3a). RNAi targeting *cnc* during embryogenesis results in transformation of the mandible to maxilla, indicating that this gene plays an important role in specifying mandible identity (Fig. 5.3b; Coulcher and Telford 2012). Expression of *cnc* is restricted to the mandible segment and labrum across mandibulate euarthropods. By contrast, it is expressed broadly across the developing embryo of chelicerates (Sharma et al. 2014) and onychophorans (Janssen 2017a). These results support a model in which the mandible characteristic of Mandibulata evolved by specialization of *cnc* function in this lineage.

In contrast to its function during embryogenesis, *Dfd* does not appear to be required for establishing mandible identity during metamorphosis in *T. castaneum* (Smith and Jockusch 2014). Instead, targeting *Dfd* during this period results in minor defects in mandible morphology but does not affect the identity of this appendage type. A similar result was recovered from studies of the postembryonic function of *Dfd* in a hemimetabolous insect species, the termite *Nasutitermes takasagoensis* (Toga et al. 2013). In this species, male minor workers can molt into either presoldiers or medium workers. The mandibles regress in size between the male minor worker and presoldier molt. When *Dfd* is targeted with RNAi, mandible regression is inhibited, i.e., presoldiers of *Dfd* RNAi treatments have larger mandibles than presoldiers of control treatments (Toga et al. 2013). This result suggests that *Dfd* functions to determine the size of presoldier mandibles postembryonically. As with postembryonic *Dfd* RNAi in *T. castaneum*, mandible identity is not affected by postembryonic *Dfd* RNAi in *N. takasagoensis* (Toga et al. 2013).

Oncopeltus fasciatus are true bugs (Hemiptera), and like other true bugs, they exhibit highly derived piercing-sucking mouthparts. In bugs, the mandibles and maxillae are modified into long thin stylets. The mandibles and maxillae form a piercing-sucking tube, with the mandible on the outside and the maxillae fused on the inside, with space between them for fluid to flow. The labial palps sheath and provide support to the feeding stylets. Of the Hox genes, only *Dfd* plays a role in establishing mandible identity in *O. fasciatus* (Hughes and Kaufman 2000). RNAi targeting this gene results in a transformation of the mandible to an antenna with multiple joints. The recognizable components of the ectopic antenna appear to exhibit distal antenna identity. Therefore, although bugs exhibit morphologically derived mandibles, *Dfd* functions to specify mandibular identity in the same manner as it does in insects with generalized mandible morphologies, by blocking antennal identity during embryogenesis.

5.6.2 *The Maxilla*

The Hox genes *pb* and *Dfd* are both expressed in the developing insect maxilla of most species that have been investigated (Fig. 5.3a; Brown et al. 1999a; Shippy et al. 2000; Curtis et al. 2001; Hughes and Kaufman 2002b; Angelini et al. 2005), and *Scr* is expressed in the maxillae of some insects that have been investigated (Passalacqua et al. 2010). Several null *pb* mutations cause nearly complete transformations of maxilla to leg in the homozygous state during embryogenesis in *T. castaneum* (Beeman et al. 1993; Shippy et al. 2000). Severely affected larvae of embryonic RNAi treatments targeting *pb* also exhibit nearly complete transformations of maxilla to leg (Shippy et al. 2000). Both loss-of-function *pb* mutations and larval RNAi targeting *pb* in *T. castaneum* also lead to transformations of the maxillae to leg during metamorphosis (Beeman et al. 1989; Smith and Jockusch 2014). In this case, only the palps are transformed, and they exhibit transformation to distal leg (femur, tibia, tarsus, pretarsus, claw). Together, these results suggest that *pb* played an ancient role in specifying maxilla identity in insects (Fig. 5.3b).

One might expect that the maxillae would develop into mandibles in the absence of *pb* function in *T. castaneum*. After all, in the absence of *pb* function, *Dfd* is the only Hox gene predicted to be expressed in the maxillae, and *Dfd* is required for specification of mandible identity (see above). Yet, the maxillae are transformed into legs when *pb* function is disrupted. This result can be explained by the fact that *cnc* is required for mandible development, and unlike *Dfd*, this gene is expressed in the developing mandibles, but not the maxillae (Fig. 5.3a; Coulcher and Telford 2012). However, *Dfd* does play an important role in maxillae development. In *T. castaneum*, *Dfd* loss-of-function embryos exhibit the telopodite component of the maxilla but lack the endite component (Fig. 5.3b; Brown et al. 2000). This suggests that *Dfd* is required for development of maxillary endites. When both *Dfd* and *pb* function are simultaneously disrupted, the maxilla develops into an antenna (Brown et al. 2002). Disrupting the function of *Dfd* and *Scr* simultaneously also results in maxilla to antenna transformations (Brown et al. 2002). The mechanism behind this result is unclear, but it most likely indicates that Dfd normally activates *pb* expression in the maxilla, but Scr can compensate for this function in the absence of *Dfd* function (Fig. 5.3a; Brown et al. 2002). In this model, when both *Dfd* and *Scr* function are compromised, *pb* is not expressed, resulting in transformation of the maxilla to antenna. There is some merit to this idea since Scr is required to activate *pb* expression in the labium of *T. castaneum* embryos (DeCamillis et al. 2001). This model of maxilla identity specification leaves open an interesting question. How does Scr affect expression of *pb* in the maxilla, since Scr is not expressed in the maxilla of *T. castaneum* embryos (Passalacqua et al. 2010)? It is possible that Scr is expressed in the maxilla when *Dfd* function is compromised, due to an inhibitory regulatory interaction between *Dfd* and *Scr*, but this possibility has not been tested in *T. castaneum*.

During *T. castaneum* metamorphosis, the roles that *pb* and *Dfd* play in maxilla identity specification are similar to their roles during embryogenesis (Smith and

Jockusch 2014). However, as with mandible development, it appears that slightly different mechanisms are active during metamorphosis. First, disrupting *Dfd* function with RNAi does not delete maxillary endites (Smith and Jockusch 2014), although this result is predicted based on studies of embryogenesis (Brown et al. 2000). Second, targeting *Dfd* and *Scr* simultaneously with RNAi does not cause homeotic transformations of the maxilla (Smith and Jockusch 2014), while the embryonic model predicts that this treatment should result in transformations of the maxillae to antenna (Brown et al. 2002). The simplest explanation for this difference is that, unlike during embryogenesis, *pb* expression does not require activation by Dfd or Scr in the maxilla during metamorphosis (Smith and Jockusch 2014).

Functional data and expression data make it clear that *pb* played a primary role in specifying maxilla identity in the last common ancestor of insects (Rogers et al. 2002). Intriguingly, however, *pb* is not expressed in the developing maxillae of the milkweed bug *O. fasciatus*, nor is this gene required for specification of maxilla identity in this species (Hughes and Kaufman 2000; Rogers et al. 2002; Angelini et al. 2005). In fact, the mechanisms that specify maxilla identity in *O. fasciatus* resemble those that specify mandible identity (Hughes and Kaufman 2000; Rogers et al. 2002). These similarities in specification resemble morphological similarities—both the mandible and maxilla are long unjointed appendages in *O. fasciatus* and other true bugs. By contrast, in other insect species, the maxilla is morphologically much more similar to the labium. Therefore, the loss of *pb* function in the maxilla of true bugs correlates with the evolution of the maxilla in this lineage toward a mandible-like morphology (Hughes and Kaufman 2000; Rogers et al. 2002). This change in morphology coupled with the loss of gene expression recalls the loss-of-function homeotic transformation of body segments that can be produced in Hox mutations in fruit flies and other animals. This correlation has led some authors (Rogers et al. 2002) to tentatively suggest that hemipteran mouthparts represent the success of a hopeful monster (Gould 1977; West-Eberhard 2003), the rare case in which a mutation of large phenotypic effect is favored and fixed by natural selection.

5.6.3 The Labium

The Hox genes *pb* and *Scr* are both expressed in the developing insect labium (Fig. 5.3a; Hughes and Kaufman 2000; Shippy et al. 2000; Curtis et al. 2001; DeCamillis et al. 2001; Hughes and Kaufman 2002b; Rogers et al. 2002; Angelini et al. 2005; Zhang et al. 2005; Hrycaj et al. 2010; Passalacqua et al. 2010). Structurally, the labium is very similar to the maxillae—consisting of basal podomeres with endites and terminal palps. However, unlike in the maxillae, the contralateral basal podomeres and endites are fused medially in the labium. Mirroring their morphological similarities, very similar mechanisms specify the maxillary and labial identities. For instance, as with the maxillae, disrupting *pb* function leads to transformations of the palps of the labium to distal leg in insect species that have been investigated (Pultz et al. 1988; Beeman et al. 1993; Hughes and Kaufman 2000; Smith and Jockusch 2014). These results indicate that *pb* plays a primary role in insects in promoting palp morphology during development. In contrast to the typical developing insect maxilla, *Scr* is typically strongly expressed in the developing labium (Fig. 5.3a; Hughes and Kaufman 2000; Curtis et al. 2001; DeCamillis et al. 2001; Rogers et al. 2002; Zhang et al. 2005; Hrycaj et al. 2010; Passalacqua et al. 2010). Therefore, *Scr* may be playing specific roles in distinguishing the labium from the maxillae. It is difficult to test this possibility during embryogenesis because *Scr* function is typically required for expression of *pb* in the labium (Fig. 5.3b; DeCamillis et al. 2001; Angelini et al. 2005). Loss of *Scr* function leads to loss of *pb* function, and the labium develops into antennae (Curtis et al. 2001; DeCamillis et al. 2001). Therefore, discriminating between *Scr* specific functions and functions of *Scr* that are mediated through its role in regulating *pb* expression are difficult in studies of insect embryogenesis. However, *Scr* does not appear to regulate *pb* expression during *T. castaneum* metamorphosis (see above). When *Scr* is targeted with RNAi during metamorphosis, the labial palps and endites develop characteristics that are typically restricted to the maxillae (Smith and Jockusch 2014). This result supports a role for *Scr* in promoting labium specific morphologies, while *pb* might play a more generic role in promoting the development of palp containing appendages.

5.6.4 The Role of Homothorax and Extradenticle in Specifying Mouthpart Identities

The protein products of genes *homothorax* (*hth*) and *extradenticle* (*exd*) must come together in the cytoplasm and form a heterodimer in order to be transported to the nucleus, where they function, in tandem, as transcription factors (Abu-Shaar and Mann 1998; Abu-Shaar et al. 1999; Kurant et al. 1998; Pai et al. 1998; Rieckhof et al. 1997). Therefore, the developmental functions of these genes perfectly overlap. Disrupting the function of either *hth* or *exd* results in homeotic transformations of gnathal appendage identities in *Gryllus bimaculatus* (Ronco et al. 2008), *O. fasciatus* (Angelini and Kaufman 2004), *Onthophagus taurus* (Simonnet and Moczek 2011), and *D. melanogaster* (Rauskolb et al. 1995; Inbal et al. 2001). These transformations most likely reflect the fact that Hth and Exd act as cofactors for Hox proteins and, as such, influence the specificity of Hox proteins for DNA regulatory elements (Chang et al. 1995; Chan et al. 1996; Johnson et al. 1995). In the absence of either Hth or Exd, Hox proteins are unable to properly regulate gene expression. This explains why the resulting phenotypes when *hth* or *exd* function is disrupted phenocopy the results of experiments in which Hox gene function is disrupted. Therefore, the roles that *hth* and *exd* play in specifying gnathal appendage identities are most likely mediated through direct interactions of their corresponding proteins with Hox proteins.

5.6.5 A General Model of Gnathal Appendage Identity Specification

Based on studies that began with *D. melanogaster* but have since expanded across diverse insects, it appears that highly conserved mechanisms control appendage identity specification in insects. The identities of most ventral appendages, including gnathal appendages, are determined by the Hox genes that are expressed in them (Hughes and Kaufman 2002a; see above). This is true for all ventral appendages except for the antennae. Hox genes are not expressed in the antennal segment (Fig. 5.3a; Hughes and Kaufman 2002a). In the absence of Hox gene function in the developing antennae, *hth* and *exd* promote antennal identity in insects (Fig. 5.3b; Struhl 1982a; Casares and Mann 1998, 2001; Mito et al. 2008; Ronco et al. 2008; Moczek and Rose 2009; Smith et al. 2014a; Setton et al. 2017). Antennal identity is specified by these genes, at least in part, by positively regulating the expression of the bHLH-PAS family transcription factor-coding gene *spineless* (Struhl 1982b; Duncan et al. 1998; Dong et al. 2002; Emmons et al. 2007; Shippy et al. 2009; Angelini et al. 2009; Toegel et al. 2009; Smith et al. 2014a; Setton et al. 2017). In developing legs, Hox genes repress *ss* expression (Duncan et al. 2010). In the absence of Hox gene activity, all ventral appendages develop as antennae (Struhl 1982a; Casares and Mann 1998, 2001; Brown et al. 2002; Smith and Jockusch 2014). While this might suggest that antennal identity is the default state of developing appendages, this is not the case. Disruption of *hth*/*exd* results in transformations of antenna to leg, even in the absence of Hox gene activity (Casares and Mann 2001; Dong et al. 2002; Ronco et al. 2008; Smith et al. 2014a). This suggests that leg identity is the default identity for ventral appendages (Fig. 5.3b; Casares and Mann 2001). To summarize the current model of ventral appendage identity specification, leg identity is most likely the default state, *hth*/*exd* promotes antennal identity in the absence of Hox gene activity, and Hox genes promote specific gnathal and leg identities combinatorially by suppressing antennal identity and the identities of other appendage types and/or by promoting particular ventral appendage identities (Fig. 5.3b).

Several features of the insect appendage identity specification mechanism predate the origin of insects. The Hox genes that pattern the gnathal appendages exhibit remarkably conserved expression patterns across Panarthropoda (Damen et al. 1998; Telford and Thomas 1998; Jager et al. 2006; Janssen and Damen 2006; Eriksson et al. 2010; Sharma et al. 2012; Janssen et al. 2014; Smith et al. 2016). Additionally, Hox genes are not expressed in the deutocerebral segment—the segment that houses antennae in insects—in Arthropoda or Onychophora (Damen et al. 1998; Telford and Thomas 1998; Jager et al. 2006; Janssen and Damen 2006; Eriksson et al. 2010; Sharma et al. 2012; Janssen et al. 2014). This suggests that specification of the appendage type that is associated with the deutocerebral segment without input from Hox genes is an ancient feature within Panarthropoda. Furthermore, RNAi targeting *hth* results in homeotic transformations of chelicerae—the deutocerebral appendages of Chelicerata—to leg in the harvestman *Phalangium opilio* (Sharma et al. 2015).

This indicates that *hth* was required for specification of deutocerebral appendage identity in the last common ancestor of Euarthropoda. Taken together, these results indicate that interactions among Hox genes and between Hox genes and *hth* were important for specifying appendage identities—including those of direct homologs of the insect gnathal appendages—in stem group Euarthropods and possibly earlier.

5.7 Developmental Genetic Patterning of Insect Appendages

While components of the core character identity network, such as Hox genes, establish the fate of different appendages, these genes activate a set of downstream genes and developmental events that direct the morphogenesis of the unique appendage types. Some of the genes involved have expression patterns and interactions that are similar across appendage types, while many are specific to the identity of the appendage. Most of our knowledge of this phase of appendage patterning comes from *D. melanogaster* and particularly from the leg imaginal disc. However, some studies in the fruit fly and other insects have examined patterning in diverse appendages, such as the mouthparts. Before considering the development of mouthparts, it will be useful to reflect on the thematic pattern demonstrated by development in the legs of insects. Several detailed reviews on the developmental genetics of insect appendages exist (Angelini and Kaufman 2005; Jockusch and Smith 2015; Jockusch 2017; Ruiz-Losada et al. 2018). Readers interested in an authoritative account of the developmental genetics of insect appendages should refer to Jockusch and Smith (2015).

5.7.1 Initiation of Appendage Primordia

The cells that are competent to give rise to ventral appendages are specified at the anterior-posterior parasegment boundaries (Estella et al. 2003). In *D. melanogaster*, cells adjacent to the posterior of the boundary express the secreted protein Hedgehog (Hh) (Ingham 1993). To the anterior, Hh induces production of secreted Wingless (Wg), in ventral cells, and Decapentaplegic (Dpp), in dorsal cells (Basler and Struhl, 1994). The areas of *wg* and *dpp* expression maintain mutually repressive interactions, reinforcing their identities (Jiang and Struhl 1996; Theisen et al. 1996). The appendage primordia ultimately inherit cells from each compartment and the expression of these segment polarity genes marking their boundaries (Diaz-Benjumea et al. 1994; Theisen et al. 1996).

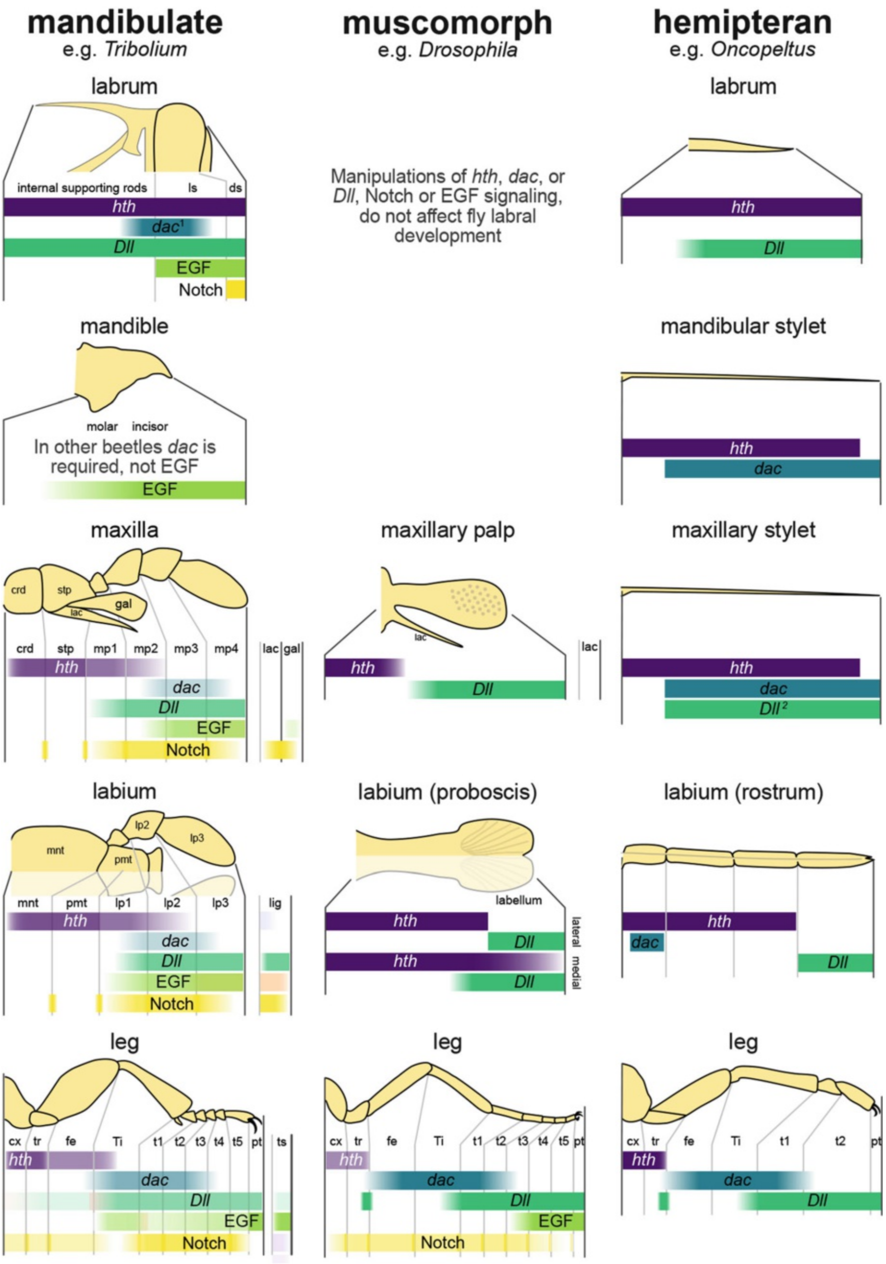
Outside of *Drosophila*, it is unclear whether these signaling pathways also initiate the expression of appendage development genes. The expression pattern of *wg* is known to extend laterally into the nascent appendages in diverse species, including the mayfly *Ephoron leukon* (O'Donnell and Jockusch 2010), the orthopterans *G. bimaculatus* (Niwa et al. 2000) and *Schistocerca americana* (Jockusch et al. 2000), the milkweed bug *O. fasciatus* (Angelini and Kaufman 2004), and the flour beetle *T. castaneum* (Bolognesi et al. 2008). However functional tests of *wg* in *G. bimaculatus* (Miyawaki et al. 2004) and *O. fasciatus* (Angelini and Kaufman 2004) appendage development do not produce defects in appendage growth or patterning. In the *T. castaneum* embryo, *wg* RNAi prevents appendage initiation (Ober and Jockusch 2006), suggesting that Wnt activation of appendage development may have evolved within Holometabola.

The transcription factor *Distal-less* (*Dll*) is one of first genes to be activated in the appendage primordia. In *D. melanogaster*, Wg promotes the expression of *Dll*, and its expression is restricted to a ventral-lateral domain in each embryonic body segment by inhibition from Dpp, dorsally, and epidermal growth factor (EGF), ventrally (Goto and Hayashi 1997). A subset of cells at the dorsal part of the Dll-expressing embryonic leg primordia contribute to the wing and haltere imaginal discs (Requena et al. 2017). In *Drosophila*, once the imaginal disc has formed, the initiation and maintenance of *Dll* expression is regulated by two separate enhancers. The first element is activated only by high levels of Wg and Dpp. Subsequently, an autoregulatory element is activated by Dll, independent of input from Wg or Dpp (Estella et al. 2008).

Dorsal-ventral specification within the leg imaginal disc is also controlled, independently, by Dpp and Wg (Estella and Mann 2008; Svendsen et al. 2009). These signaling molecules activate expression of transcription factors encoded by *optomotor blind* (*omb*) and *H15* in dorsal and ventral territories, respectively (Maves and Schubiger 2003; Wilder and Perrimon 1995). Orthologs of *omb* and *H15* are expressed in similar dorsal and ventral territories in the limb buds of the pill millipede *Glomeris marginata* (Prpic et al. 2005), but the expression of *H15* is reduced in the spider *Cupiennius salei* (Prpic et al. 2003) and actually appears in a dorsal area of the limb buds in the onychophoran *Euperipatoides kanangrensis* (Janssen et al. 2015). Moreover, patterns of *wg* and especially of *dpp* expression do not conform with the *Drosophila* model in most other arthropod species (Angelini and Kaufman 2005; Janssen et al. 2015). These results suggest that, while the specification of dorsal-ventral polarity may be conserved within insects, its establishment may rely on as yet unidentified factors.

5.7.2 Specification of Proximal-to-Distal Domains

By the late second instar, gene expression begins to differentiate discrete domains along the proximal-to-distal axis of the *Drosophila* leg imaginal disc (Lecuit and Cohen 1997), and similar patterns have been found in other insects (Fig. 5.4; Angelini and Kaufman 2005; Jockusch and Smith 2015). High levels of both Wg and Dpp occur only in the center of the *D. melanogaster* leg imaginal disc, where cells expressing the two signals are near each other spatially along the parasegment



Manipulations of *hth*, *dac*, or *Dll*, Notch or EGF signaling, do not affect fly labral development

In other beetles *dac* is required, not EGF

Fig. 5.4 Summary of the requirement for appendage-patterning genes in the development of three insect species with different mouthpart morphologies. Distal structures are to the right in each panel, and lateral is up, except in the diagrams of legs where dorsal is up. Colored bars highlight structures affected by the manipulation of *hth*, *dac*, *Dll* and different components of the EGF and Notch signaling pathways. Notes: 1. While *dac* is expressed in an intermediate domain of the embryonic *T. castaneum* labrum (Prpic et al. 2001), embryonic *dac* RNAi has not been reported.

boundary (Lecuit and Cohen 1997; Wu and Cohen 1999). In this way, *Dll* expression becomes locked in at the center of the leg disc, where its activity is required for development of the telopodite, the distal region of the leg (Cohen and Jürgens 1989b).

While the establishment of the proximal-to-distal axis by dorsal and ventral gradients of *dpp* and *wg* expression has been well described in *D. melanogaster*, a comparable model is lacking for insects generally. Prpic et al. (2003) have argued that this model of *Dll* activation, in the context of a roughly two-dimensional imaginal disc, does not generalize to the three-dimensional limb buds that are ancestral to insects and other arthropods. These authors point out that, because *dpp* and *wg* are expressed along dorsal and ventral sides of the compartment boundary, their secreted products form two hyperbola-shaped domains that intersect only at the center of the disc. However, if the same model is generalized to three dimensions, then cells along the length of the limb would experience similar concentrations of signaling proteins produced from the dorsal and ventral sides. This theoretical consideration helps to explain the diversity of *dpp* expression patterns that have been found (Angelini and Kaufman 2005; Janssen et al. 2015). However, it is still not clear what mechanism produces unique proximal-to-distal gene expression outside of *Drosophila* for genes such as *Dll*.

5.7.3 Proximal-Distal Domain Genes: Distal-less, Dachshund, and Homothorax

Genes such as *Dll* have been dubbed "limb gap genes" because their loss-of-function phenotype eliminates structures from the limb and reduces growth of cells in those areas. This name is an analogy to the gap genes involved in *Drosophila* embryonic germ band patterning, where mutations in gap genes produce similar phenotypes (Nüsslein-Volhard and Wieschaus 1980; Wieschaus and Nüsslein-Volhard 2016). *Distal-less* is expressed in the *D. melanogaster* leg disc in cells that will give rise to the distal tibia and tarsus (Lecuit and Cohen 1997; Panganiban and Rubenstein 2002). A narrow ring of *Dll* expression also appears in the distal trochanter shortly before pupation (Wu and Cohen 1999). Strong *Dll* loss-of-function alleles in *D. melanogaster* are embryonic recessive lethal (Sunkel and Whittle 1987), but hypomorphic alleles or imaginal discs with reduced or eliminated *Dll* activity cause the loss of distal structures from the leg, including the femur, tibia, and tarsus (Cohen

Fig. 5.4 (continued) Metamorphic-stage *dac* RNAi does not produce noticeable defects in the labrum (Smith et al. 2014). 2. *Dll* is expressed in the embryonic maxillary appendages in *O. fasciatus*, but *Dll* RNAi has no noticeable effect on their development. *crd* cardo, *cx* coxa, *ds* distal sclerite of the labrum, *fe* femur, *gal* galea, *lac* lacinia, *lig* ligula (single labial endite), *lp1–3* labial palp segments 1–3, *ls* labral sclerite, *mnt* mentum, *mp1–4* maxillary palp segments 1–4, *pmt* prementum, *pt* pretarsus, *stp* stipes, *t1–5* tarsomeres 1–5, *Ti* tibia, *tr* trochanter, *ts* tibial spurs and Jürgens 1989b). The expression pattern of *Dll* orthologs is well conserved in the distal legs of diverse insects and other animals (Jockusch and Smith 2015). Mutations or RNA interference reducing *Dll* activity has also produced deletion of the legs, distal to the trochanter, in several hemi- and holometabolous insect species (Fig. 5.4; Beermann et al. 2001; Angelini and Kaufman 2004; Ohde et al. 2009; Yoshiyama et al. 2013; Angelini et al. 2012b; Moczek and Rose 2009).

The proximal domain of the insect leg is marked by expression of the homeobox transcription factor *homothorax* (*hth*). Wg and Dpp act to inhibit the expression of *hth* in central parts of the leg imaginal disc, restricting its expression to the periphery (Abu-Shaar and Mann 1998; Wu and Cohen 1999). This pattern of *hth* expression in developing legs appears conserved in many insects (Prpic et al. 2003; Angelini and Kaufman 2004; Inoue et al. 2002) and in other arthropods (Prpic and Tautz 2003). In *D. melanogaster*, Hth functions by binding with its cofactor encoded by *extradenticle* (*exd*; Abu-Shaar and Mann 1998; Rieckhof et al. 1997). Leg imaginal discs that lack *hth* develop with a fusion of proximal leg structures, aberrant joint formation, or a proximal-to-distal transformation of podomeres (Casares and Mann 1998, 2001). A similar leg phenotype is found with *hth* or *exd* RNAi in *O. fasciatus* (Fig. 5.4; Angelini and Kaufman 2004), *G. bimaculatus* (Mito et al. 2008; Ronco et al. 2008), and *T. castaneum* (Smith and Jockusch 2014).

A unique intermediate domain becomes established later in the second instar leg imaginal disc with the expression of *dachshund* (*dac*) (Mardon et al. 1994; Giorgianni and Mann 2011). Over time, the area of *dac* expression expands to encompass cells that will give rise to the femur, tibia, and basitarsus. As with the activation of *Dll*, Wg and Dpp promote the expression of *dac* in the *D. melanogaster* leg imaginal disc (Lecuit and Cohen 1997). Its area of expression is refined through co-activation by Brinker (Brk), which is expressed in areas of the disc outside the influence of Dpp (Estella and Mann 2008). Dll also directly binds to a *dac* regulatory element to initiate its expression (Giorgianni and Mann 2011). Later in the third instar, *Dll* and *dac* distinguish the distal and intermediate domains of the leg through mutually antagonistic interactions (Dong et al. 2001). Orthologs of *dac* are expressed in similar patterns in the developing legs of diverse insects (Abzhanov and Kaufman, 2000; Schaeper et al. 2013; Inoue et al. 2002; Prpic et al. 2001; Angelini and Kaufman 2004; Tanaka and Truman 2007), although some differences exist among taxa in the dynamics and precise proximal or distal limits of *dac* expression (Jockusch and Smith 2015). Mutations eliminating *dac* activity in *D. melanogaster* reduce the length of the leg by eliminating the tibia, giving this gene its name in reference to the short-legged dog breed. Maternal RNAi in *O. fasciatus* produces embryos with similar deletion of the tibia (Fig. 5.4). Surprisingly, *dac* RNAi in *T. castaneum* embryos produces only minor leg defects (Lee et al. 2013), although RNAi during metamorphosis in the species results in deletion of the tibia (Angelini et al. 2012b), similar to the *D. melanogaster dac* mutant phenotype (Fig. 5.4).

Studies in diverse insects have largely supported the conservation of *Dll*, *dac*, and *hth* in establishing the pattern of proximal-to-distal domains in the leg. While small differences in the precise limits of expression and in timing exist (reviewed by Jockusch and Smith 2015), the homology of this network within leg development seems certain. In *Drosophila*, the interactions that define expression boundaries between the proximal-to-distal domain genes have been examined through elegant clonal analysis studies. Using methods for timed mosaic generation of cells with deletion alleles (Xu and Rubin 1993; Lee and Luo 1999), it is possible to see how cells lacking, for example, a distal gene change their expression of other genes or interact with neighboring wild-type cells. Using these methods, it has been found that the three principal proximal-distal domain genes, *Dll*, *dac*, and *hth*, interact antagonistically in a way that helps define each area (Dong et al. 2001; Wu and Cohen 1999).

The initial pattern established by *Dll*, *dac*, and *hth* is elaborated as other genes also become expressed in the leg, directing smaller aspects of local identity (reviewed by Angelini et al. 2012b; Jockusch and Smith 2015). The distal segmentation of the tarsus and development of the pretarsal structures are controlled by EGF signaling in *D. melanogaster* (Campbell 2002; Galindo et al. 2002). This terminal appendage-patterning role for EGF appears to be widely conserved. Knockdown of the EGF ligand during metamorphosis also eliminated the tarsus and tibial spurs in the legs of *T. castaneum* (Grossmann and Prpic 2012; Angelini et al. 2012b). Similarly, RNAi targeting the EGF receptor prevented regeneration of the distal tarsus and pretarsus in the legs of *G. bimaculatus* (Nakamura et al. 2008). Another well-conserved aspect of later appendage development is the requirement for Notch signaling in joint formation. In *D. melanogaster*, the Notch ligands Delta and Serrate are expressed adjacent to the locations of joint formation (de Celis et al. 1998; Bishop et al. 1999; Rauskolb and Irvine 1999; Tajiri et al. 2011), and the terminal EGF signal helps determine the position of joints in the leg by regulating the expression Notch pathway genes (Galindo et al. 2005). The role of Notch signaling in joint formation has been confirmed by RNAi in the insects *G. bimaculatus* (Mito et al. 2011) and *T. castaneum* (Angelini et al. 2012b). The spider *Cupiennius salei* also requires Notch signaling activity for leg growth and joint formation, leading to the suggestion that this function is an ancestral and defining feature of all euarthropods (Prpic and Damen 2009).

5.8 Developmental Genetic Patterning of Mandibulate Mouthparts

The developmental patterning of mouthparts is similar in many ways to the theme represented by legs. Unique morphologies are reflected by variations in the developmental system. Mandibulate mouthparts are the ancestral state for insects (Snodgrass 1935), but they also bear the closest resemblance to the theme established by leg development (Angelini et al. 2012a). The development of mandibulate mouthparts has been investigated through functional genetic tests in hemimetabolous and holometabolous species, including the primitively wingless insect *Thermobia domestica* (Schaeper et al. 2013), the cricket *G. bimaculatus* (Ronco et al. 2008), the beetles *Onthophagus taurus* (Simonnet and Moczek 2011) and *Tribolium castaneum* (Angelini et al. 2012a), and the stag beetle *Cyclommatus metallifer* (Gotoh et al. 2017).

5.8.1 The Mandible

The mandible is the most anterior gnathal appendage, and it is unique in many ways. The insect mandible is unjointed, consisting of a single heavily muscled segment. The relative simplicity of its anatomy and its resemblance to the proximal-most segments of other appendages gave rise to the suggestion that the insect mandible is homologous only to other proximal appendage segments (Snodgrass 1935; Kukalová-Peck 1998). However it has also been suggested that the mandible evolved by reduction and elimination of joints, essentially retaining homology with the full proximal-to-distal extent of other appendages (Manton 1964). The gnathobasic hypothesis has been supported by developmental genetic studies of the distal appendage gene *Dll*, which is not expressed in the mandibles in insects (Panganiban et al. 1994; Scholtz et al. 1998; Popadić et al. 1998), and its suppression by RNAi does not affect mandible development (Niimi et al. 2005; Moczek and Rose 2009; Beermann et al. 2001; Angelini et al. 2012a; Gotoh et al. 2017; Yoshiyama et al. 2013). In *T. castaneum*, the Hox gene *Dfd* activates expression of *cnc* in the mandibular body segment, which inhibits expression of *Dll* (Coulcher and Telford 2012).

However, studies of different beetle species have revealed diverse roles for other genes in shaping the mandible. A functional study of 13 candidate appendage-patterning genes in the tenebrionid *T. castaneum* identified a role for EGF signaling in the mandible (Fig. 5.4; Angelini et al. 2012a). *EGF* RNAi significantly reduced mandible length, reducing the medial-distal incisor area in the flour beetle. This finding was unexpected, since EGF is required for formation of distal leg structures in diverse insects, including *G. bimaculatus* (Nakamura et al. 2008), *T. castaneum* (Grossmann and Prpic 2012; Angelini et al. 2012b), and *D. melanogaster* (Campbell 2002; Galindo et al. 2002). RNA interference targeting other appendage-patterning genes, including *dac* and *hth*, did not produce defects in the mandible of *T. castaneum.* In contrast, studies in scarabaeoid species *O. taurus* (Simonnet and Moczek 2011) and *C. metallifer* (Gotoh et al. 2017) found that RNAi suppression of *dac* caused reduction of mandibular teeth or incisors. Male *C. metallifer* have enlarged mandibles, and *dac* RNAi also significantly reduced their growth. Both studies also identified unique aspects of mandible patterning in these species. Depletion of *hth* modified a ridge between the molar and incisor regions in *O. taurus* (Simonnet and Moczek 2011) and eliminated the development of the medial mandibular teeth in *C. metallifer* (Gotoh et al. 2017). Other genes have not yet been examined in *O. taurus*, but RNAi targeting the distal leg gene *aristaless* also eliminated the mandibular teeth in *C. metallifer* (Gotoh et al. 2017). In contrast to its prominent role in the mandible of *T. castaneum*, *EGF* RNAi in *C. metallifer* did not cause noticeable defects.

Fully evaluating the gnathobasic hypothesis will require additional functional studies of mandibulate insects, especially among early-branching insect lineages. One possibility is that, while the ancestral state for insects may be gnathobasic, the existing interactions among appendage-patterning genes, necessary for the development of other appendage types, may have facilitated the evolutionary co-option of these genes into mandible development for roles in patterning novel structures, such as mandibular teeth.

5.8.2 The Maxilla and Labium

Patterning of the maxillae and labium is similar, reflecting similarities in their morphology. Their development also requires the same proximal-distal patterning genes known from leg development (Fig. 5.4). Exactly how these and other developmental regulatory genes direct appendage-specific anatomy is not completely clear. However, a striking difference is that the extent of overlap in gene expression is much greater for *Dll*, *dac*, and *hth* in the maxilla and labium than it is in the legs (reviewed by Angelini and Kaufman 2005; Jockusch and Smith 2015). It is likely that the combination of these transcription factors, along with regulatory proteins unique to these body segments, such as specific Hox protein combinations, directs specific target genes that ultimately leads to morphogenesis of these appendage types.

Distal-less is expressed in the palps and endites of both appendage types across diverse mandibulate insects (reviewed by Angelini and Kaufman 2005; Jockusch and Smith 2015). These structures are also eliminated by *Dll* mutation or RNAi in *T. castaneum* (Beermann et al. 2001; Angelini et al. 2012a). However, some variations in the requirement of *Dll* may exist among species, since metamorphic-stage *Dll* RNAi in the dung beetle *O. taurus* does not affect development of the maxillary endites (Simonnet and Moczek 2011), although other appendages have phenotypes resembling similar experiments in *T. castaneum* (Angelini et al. 2012a). Knockdown of *Dll* by RNAi in the sawfly *Athalia rosae* (Yoshiyama et al. 2013) and in the firebrat *Thermobia domestica* (Ohde et al. 2009) also reduced the maxillary and labial palps, causing fusion of palp segments. These studies did not report potential effects of *Dll* RNAi on the medial endites.

Expression of the intermediate domain gene, *dachshund*, occurs in the second maxillary and labial appendage segments (the stipes and prementum), in the maxillary and labial endites, and in a proximal to intermediate region of the palps (reviewed by Angelini and Kaufman 2005; Jockusch and Smith 2015). Tests for the functional requirement of *dac* in the mandibulate maxilla and labium have only been reported from the beetles *T. castaneum* (Angelini et al. 2012a) and *O. taurus* (Simonnet and Moczek 2011). In both species, *dac* RNAi reduces the length of and number of joints within the maxillary palps. However, in the labium, the two species have different *dac* RNAi phenotypes. In *O. taurus*, *dac* RNAi causes reduction of the prementum, while in *T. castaneum* the *dac* RNAi phenotype is similar in the maxilla and labium, with reductions in the length and joint number in the palps. Wider phylogenetic sampling is needed, but the serial homology among gnathal appendages suggests that a *dac* function in the palps may be ancestral.

Proximal appendage genes, *hth* and *exd*, are expressed across a much larger area of the maxilla and labium than they are in the legs (reviewed by Angelini and Kaufman 2005; Jockusch and Smith 2015). This creates a large degree of overlap between genes known for proximal and distal specification in the leg. While interactions among these genes have not been tested in the maxilla or labium of mandibulate insects, we predict that they should not exhibit the same antagonism seen during *D. melanogaster* leg development (e.g., Wu and Cohen 1999; Dong et al. 2001). RNA interference targeting *hth* in *T. castaneum* causes effects across the maxilla and labium (Angelini et al. 2012a). Shape changes occur in the proximal segments that may represent transformation toward more distal identities. Endites are present but reduced. Only the distal segments of the palps are unaffected. Depletion of *hth* in *O. taurus* produced similar phenotypes although the endites and palps of the maxilla appeared normal (Simonnet and Moczek 2011). The function of *hth* has also been tested in *G. bimaculatus*; however, its depletion by RNAi caused the transformation of mouthparts toward a mixed antenna-leg identity (Ronco et al. 2008). As discussed above, Hth also functions as a cofactor for Hox protein function.

Other aspects of gnathal appendage development follow the theme set by the legs (Fig. 5.4). Joints express and require locally restricted components of the Notch signaling pathway (Mito et al. 2011; Angelini et al. 2012a), and terminal patterning genes, such as *aristaless*, are required for development of the endites and the distal tip of the palps (Miyawaki et al. 2002; Angelini et al. 2012a).

5.9 Patterning Variations in Derived Mouthpart Morphologies

A number of successful insect lineages have evolved variations on the mandibulate theme. Two representatives of such taxa have been studied at the developmental genetic level: the fruit fly *Drosophila melanogaster* and the milkweed bug *Oncopeltus fasciatus*.

5.9.1 *The Labellate Proboscis of* Drosophila

Muscomorpha have evolved an adult proboscis used to lap up liquid or semiliquid foods. In *Drosophila*, the proboscis is derived mostly from the labium, although labial palps or endites are absent. Maxillae are present on the lateral sides of the proboscis. A small maxillary base branches into a lacinia and maxillary palp. Adult mandibular structures are reduced and incorporated into the head capsule.

Signals that establish the proximal-distal axis of developing adult mouthparts are expressed in the late third instar larva, later than in the legs. Wnt and Dpp signaling is required for development of the maxillary field within the eye-antennal imaginal disc and for development of the labial imaginal discs (Joulia et al. 2005; Yasunaga et al. 2006; Doumpas et al. 2013), as they are in leg development. However, the timing of these signals is critical for the identity and patterning of both structures. If Wnt expression is activated early, the maxillary field develops as an ectopic antenna (Lebreton et al. 2008). In the labial disc, the Hox protein Pb represses *hh*, which results in reduced expression of *wg* and *dpp* (Joulia et al. 2005). In the absence of this repression, the labial disc develops as pair of ectopic legs.

Distal-less expression is activated in the developing adult maxillary field and labial imaginal disc by Wg and Dpp (Joulia et al. 2005; Yasunaga et al. 2006), although its expression is less intense than in the leg imaginal disc. *Dll* is expressed across the distal third of the labial disc (Fig. 5.4; Abzhanov et al. 2001; Joulia et al. 2005; Yasunaga et al. 2006). Mosaic mutant cells lacking *Dll* in the maxillary field fail to form maxillary palps, although the proximal base and lacinia remain (Cohen and Jürgens, 1989b). Distal structures of the labium are eliminated by loss of *Dll* mosaic clones in the labial disc (Cohen and Jürgens, 1989a; Yasunaga et al. 2006). Levels of *Dll* expression are also controlled by negative regulation from Scr (Abzhanov et al. 2001).

There appears to be no role for *dac* in the development of *Drosophila* mouthparts. The presence of Pb causes suppression of *dac*, and the maxillary field and labial disc in *D. melanogaster* show no expression of Dac (Abzhanov et al. 2001; Joulia et al. 2005). Abnormal phenotypes in the mouthparts have not been reported for *dac* loss of function.

The proximal leg patterning gene *hth* is expressed across the entire lateral layer of the labial imaginal disc, where it overlaps with *Dll*, but it is limited to the proximal two thirds as the medial layer (Yasunaga et al. 2006). Expression of *hth* occurs throughout the maxillary field (Pai et al. 1998). Exd, the cofactor required for Hth function, has very little expression in the maxillary field (Abzhanov et al. 2001). Nevertheless, loss of *hth* from the eye-antennal disc eliminates the maxillary palps (Stultz et al. 2012). The interactions among *Dll* and *hth* differ in the medial and lateral layers of the labial disc (Yasunaga et al. 2006). *Dll* represses *hth* only in the lateral layer, while the two genes are co-expressed medially. Since *Dll* and *hth* also are co-expressed in the antenna (reviewed by Angelini and Kaufman 2005; Jockusch and Smith 2015), the labium has been suggested as developing through an intermediate developmental program, rather than a completely unique one (Yasunaga et al. 2006).

5.9.2 The Rostrum of Oncopeltus

The mouthparts of Hemiptera are modified into a rostrum used for feeding by piercing and sucking liquid foods (Meek 1903; Snodgrass 1921). Different species have applied this strategy to predation and to phytophagy. One of the major differences in hemipteran mouthparts is that the difference in similarities among the gnathal appendages. While in mandibulate mouthparts the maxilla resembles the labium, in Hemiptera, it more closely resembles the mandibular appendages. However, both the mandibular and maxillary appendages of Hemiptera are modified into long, slender stylets. These interlock along their length, forming multiple channels for secretion of saliva and the uptake of liquefied food. The labial appendages are fused medially. The stylets are held in a midventral groove, and the rostrum is manipulated by the insect by means of joints between four labial segments.

The developmental genetic patterning of the hemipteran rostrum has been studied in the milkweed bug *Oncopeltus fasciatus* (Angelini and Kaufman 2004, 2005). The anatomical similarity in mandibular and maxillary appendages in *O. fasciatus* correlates with similar expression patterns in the gnathal Hox genes. In most insects, including *Drosophila* and *Tribolium*, the mandibular and maxillary body segments express the Hox gene *Dfd*, while *pb* is expressed in the maxillary and labial segments. The labial segment is also distinguished by expression of *Scr* (reviewed by Hughes and Kaufman 2002a). However, in *O. fasciatus*, the expression of *pb* is limited to the labial appendages (Rogers and Kaufman 1997; Angelini et al. 2005). As a result, the mandibular and maxillary segments develop with only Dfd for Hox regulation. RNA interference targeting the gnathal Hox genes produces transformations of the appendages in segments where these genes are normally expressed (Hughes and Kaufman 2000).

Proximal-distal domain genes also demonstrate the similarity of mandibular and maxillary development in Hemiptera (Angelini and Kaufman 2004). In embryos of *O. fasciatus*, the mandibular and maxillary limb buds both express *dac* and *hth* throughout their length. Both of these genes are also required for proper development of the stylets. Knockdown of *dac* by RNAi causes failure of the embryonic appendages to differentiate into stylets. Following *hth* RNAi, only the distal tips of the stylets differentiate, but the proximal majority of the appendage fails to and does not invaginate and coil into the head as normal (Dorn and Hoffmann 1983; Newcomer 1948). RNAi targeting *Dll* has no effect on stylet development in *O. fasciatus*, although the antennae, labium, and legs are all truncated (Angelini and Kaufman 2004). Juvenile-stage *Dll* RNAi also affects development of the male and female genitalia in *O. fasciatus* (Aspiras et al. 2011). The absence of a functional requirement for *Dll* in the hemipteran mandibular and maxillary and stylets is similar to what has been found in the development of mandibles in other insects. Unexpectedly, *Dll* mRNA and protein are expressed strongly in the maxillary limb buds of *O. fasciatus* embryos, although not in the mandibular limb buds (Rogers et al. 2002; Angelini and Kaufman 2004). This suggests two implications: First, a mechanism must exist to inhibit the function of the Dll transcription factor specifically in the maxillary body segment. Second, activation of *Dll* in the maxillary appendages is likely independent of Hox regulation, since both the mandibular and maxillary segments share the same Hox protein milieu. One possibility is activation of *Dll* by the gap gene *hunchback* (*hb*), which is expressed throughout the future head region in blastoderm-stage embryos, but *hb* expression is markedly more intense in the maxillary and labial body segments (Liu and Kaufman 2004).

The homology of hemipteran mouthpart structures to those of mandibulates has been uncertain (Meek 1903; Snodgrass 1921). Anatomists have proposed homology of the maxillary stylets to several components of the mandibulate maxilla, including the stipes (Cobben 1979), palpigers (Muir and Kershaw 1911a), lacinia (Crampton 1923; Hamilton 1981; Muir and Kershaw 1911b, 1912; Newcomer 1948; Snodgrass 1938, 1944), and to the entire maxilla (Bourgoin 1986; Parsons 1964, 1974). Perhaps this is asking the wrong question? The shift in *pb* expression and the functional similarities in *Dll*, *dac*, and *hth* in the mandibular and maxillary appendages suggests that, rather than modification of maxillary structures, the hemipteran maxillary stylets may have evolved by redeploying the mandibular developmental program within the maxillary appendages. Viewed in this way, both hemipteran stylets are homologous to the ancestral insect mandible. Moreover, the prominent functions of *dac* and *hth* in *O. fasciatus* stylet development fit well with developmental and anatomical evidence suggesting that the mandible is a proximal, gnathobasic structure.

5.10 Differences in Embryonic and Postembryonic Appendage Patterning

Appendages do not reach their final state in an individual insect until adulthood. Wings and genitalia are extreme in this regard, since they are not fully functional until adulthood. The subimago of Ephemeroptera is an exception, having functional wings in the last preadult stage (Edmunds and McCafferty 1988). However, other appendages, such as the antennae, mouthparts, and legs, appear in the juveniles of most insect groups and undergo subsequent development and repatterning during nymphal or pupal molts. Juvenile legs typically lack joints in distal structures, such as the tarsus or tibiotarsus. Experiments in beetle *Tenebrio molitor* using amputation (Huet and Lenoir-Rousseaux 1976) suggest that the entire larval leg contributes to the adult leg with cells maintaining their approximate relative position within the limb.

Once structures are formed during development, it is unclear to what extent their identity is irreversibly determined or whether they require continuous expression of genes to maintain their identity. Such a requirement may differ by species or between hemi- and holometabolous insects. The dramatic delay in appendage development in *Drosophila* has meant that the fruit fly has not provided its usual insights into development regarding differences in embryonic and postembryonic appendage patterning. Instead, other model species have provided these comparisons.

In the hemimetabolous hemipteran *O. fasciatus*, all juvenile stages have legs that closely resemble the adult in gross anatomy but have two tarsomeres to each leg. At the imaginal molt, the distal tarsomere is divided by a new joint, producing three tarsomeres in total. While the leg distal of the trochanter is lost if *Dll* is suppressed by RNAi during embryogenesis (Angelini and Kaufman 2004), only the distal tarsal joints of the adult are affected by *Dll* RNAi during the last juvenile instar (Aspiras et al. 2011). In contrast, the holometabolous species *T. castaneum* requires *Dll* activity continuously to maintain the growth and identity of leg structures. *Tribolium* adult legs have four to five tarsomeres, but larvae have a fused tibiotarsus. *Dll* mutations in *T. castaneum* affect larval and adult legs distal of the trochanter, causing reduced growth and an absence of distal identity affecting the femur, tibia, tarsus, and pretarsus (Beermann et al. 2001). When *Dll* is targeted by RNAi during the pupal stage, even structures such as the femur, which were properly formed in the same individuals as larvae, can be affected by the loss of *Dll* activity (Angelini et al. 2012b). Examination of in situ gene expression in embryonic, larval, and pupal legs of *Manduca sexta* (Lepidoptera), another holometabolous species with robust larval legs, has found a continuity of expression of *hth*, *dac*, and *Dll* at their respective proximal to distal levels in the leg as individuals undergo metamorphosis (Tanaka and Truman 2007).

Similarly, in the mouthparts of *T. castaneum*, gene activity is required to maintain the identity of specific regions during metamorphic development. Pupal-stage RNAi targeting *hth*, *dac*, or *Dll* produces defects in structures that were present in the larval maxilla and labium (Angelini et al. 2012a). While the requirement for these genes is limited to the tarsus during adult development of the legs in *O. fasciatus*, the mandibular and maxillary stylets continue to require *dac* activity for proper development during the imaginal molt (Aspiras et al. 2011), similar to its role during embryonic development (Angelini and Kaufman 2004). While the gross anatomy of the adult labium (rostrum) of *O. fasciatus* is not obviously altered by juvenile RNAi targeting proximal-distal domain genes, the length of the labium is reduced by *Dll* RNAi at this stage (Angelini and Kaufman 2005; Aspiras et al. 2011).

5.11 The Future of Research on Insect Appendage Development

Developmental genetics is still far from a detailed understanding of how genetic networks sculpt anatomy. However, we are beginning to appreciate how character identity networks initiate the development of specific structures. Mutant screens and functional analyses such as the production of mosaic discs have provided deep insights into appendage development in *D. melanogaster*. In contrast, studies of other species have relied heavily on a candidate-based approach, in which orthologs of genes from *D. melanogaster* developmental models are preferentially tested for roles in other species, highlighting instances of conservation or difference. While this path has been fruitful, it leads to a perspective of diversity that is likely biased toward conserved features of development. We often view other insects in terms of how they are "not like fruit flies" rather than how they uniquely generate their own morphologies.

Given the disparity of insect mouthparts, much remains to be learned about how genes direct this diversity of forms. Many model species are amendable to developmental and molecular genetic studies in ecologically and economically important insect groups with unique mouthparts. First among these are the Lepidoptera. A genetic model for the development of the galeate proboscis of moths or butterflies would provide important insights into this key innovation of the Lepidoptera.

Thankfully, the increasing accessibility of genomics and functional genetic manipulations is beginning to change the current situation. For example, a recent study of water striders used transcriptome comparisons among different legs to identify novel genes associated with the evolution and development of a unique, taxon-specific fan structure at the distal end of the midleg (Santos et al. 2017). RNA interference enabled tests of the gene's requirement in fan development, as well as the fan's function in the insect's locomotion. Similar applications of genomic methods should enable more sophisticated approaches that are not constrained by the assumptions of conservation with traditional genetic model species.

5.12 Returning to the Theme of Homology

Focusing on the genes responsible for development of traits underscores both special homology (inheritance from a common ancestor) and serial homology (deployment at multiple locations across the body plan). Importantly, this concept can also contextualize anatomical themes and variation seen across organisms. As we have recounted, some aspects of the developmental network may be conserved among serially homologous structures, such as the specification of appendage identity by Hox genes and the requirement of *Dll* for development of distal appendage structures, while other aspects, such as local interactions among patterning genes, may vary.

Importantly, serial homologs are not evolutionarily independent (Wagner 2007; Angelini et al. 2012b; Jockusch and Smith 2015). Serial homologs share developmental mechanisms and the genes that comprise their components. In this way, they have a shared evolutionary history, by virtual of their common genes, and a shared developmental history, via redeployment of those genes in different locations. Nevertheless, serial homologs can experience different selection pressures and may therefore evolve independently over time. Mutations in different lineages may affect development in ways that are general or specific with regard to serial homologs (Fig. 5.5). We will term evolutionary change causing uniform, similar changes

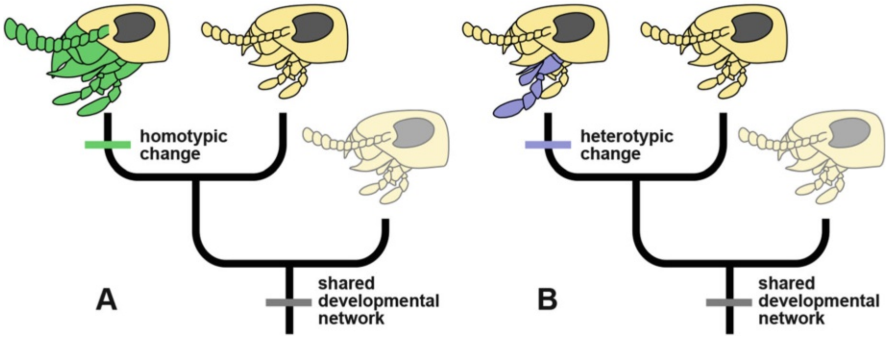


Fig. 5.5 Serial homologs may evolve in concert, via homotypic change, or independently, via heterotypic change. These differing types of evolution are depicted here for mouthparts of a generic beetle-like insect. (**a**) In homotypic change, all serial homologs, such as the appendages, are affected pleiotropically and exhibit similar changes compared to the ancestral state. In this example, all appendages become enlarged and green. (**b**) In contrast, heterotypic change is limited to one serial homolog. In this example, the maxilla increases in size and changes color to blue. We predict mutations causing heterotypic change to be qualitatively different, such that their effects are limited in scope. The most likely mechanism for this specificity is change in a gene's regulatory elements, controlling expression in a given region

in all serial homologs homotypic change, while a change affecting a subset of serial homologs may be known as a heterotypic change.

With some understanding of the developmental system, we might begin to ask, what kinds of mutations are likely to result in homotypic or heterotypic change? An intuitive hypothesis might focus on the distinction between "core" genes, which function early and upstream in the developmental network in all serial homologs, and homolog-specific genes function later and downstream to effect unique morphology (Davidson 2006). This model would predict homotypic changes would result from mutations in the "core" genes and heterotypic changes would result from mutations in the more downstream, homolog-specific genes. However, our understanding of insect appendage development does not support this hypothesis (Fig. 5.4).

From what is known of appendage development, serial homologs differ from one another in their development at all levels. A comparison of *D. melanogaster* labial and leg imaginal discs helps illustrate this point. In both appendages, specification of the proximal-distal axis is accomplished by Wg and Dpp gradients, but this is indirectly modulated by *pb* activity in the labial disc in a way that is essential for labial development (Joulia et al. 2005; Yasunaga et al. 2006). Downstream of axis specification, the proximal-distal domain genes *Dll* and *hth* are co-expressed in the medial labial disc, but not laterally, as in the leg (Yasunaga et al. 2006). Similarly, *Dll*, *dac*, and *hth* are all expressed in antennae, mouthparts, and legs of diverse insects, but their areas of overlap differ (Fig. 5.4).

Rather than fixing mutations at different levels of the regulatory hierarchy, heterotypic evolution of serial homologs appears to have proceeded through changes in the regulatory interactions among genes that are part of a common theme in appendage development. Therefore, we predict that the key genetic differences underlying the unique morphologies of serial homologs, as well as the sites of mutation affecting their evolution, will be found in the regulatory elements of genes required for their formation (Rebeiz and Tsiantis 2017). If it is correct that morphological evolution among species proceeds more often via regulatory changes (Stern and Orgogozo 2008; Stern 2010), then similarities between serial homologs are likely to be retained to some extent by pleiotropy.

Additional developmental genetic studies are necessary to fully test this hypothesis. While technically demanding, functional tests of regulatory elements will be needed in a wider diversity of insect models. Genome editing technologies are beginning to make this possible. Ultimately, our goal is to read the musical genetic notation and understand how variations in morphological diversity emerge from the theme.

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