3 The Role of Hox Genes in the Origins and Diversification of Beetle Horns

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3.1 INTRODUCTION

The discovery of Hox genes and their remarkable degree of conservation and organization transformed our understanding of the origins of animal diversity. It marked the beginning of an intellectual journey away from organisms and their component parts as uniquely constructed by taxon-specific information and toward animal diversity as arising from conserved, deeply homologous building blocks (Carroll, Grenier, and Weatherbee 2005). Initially, this deep level of conservation posed a challenge to our understanding of the origins of phenotypic diversity: if genes, their products, and the pathways they contribute to are so conserved, how could evolution not be severely constrained? This challenge was overcome through the realization that development is also extraordinarily modular across levels of biological organization. Consequently, relatively simple evolutionary changes in the location, timing, amount of product, and governance of developmental processes were found to be sufficient to beget tremendous diversity, emerging not *despite* the conservation of developmental building blocks, but *because* of it (reviewed in Moczek 2019).

The revolution initiated by the discovery of Hox genes also forced a reassessment of our understanding of the origins of novelty in evolution. Prior, evolutionary novelty was considered to start when homologous relationships ended. In fact, with respect to *morphological* novelties, one of the most commonly used definitions explicitly requires the absence of homology and homonomy: "A morphological novelty is a structure that is neither homologous to any structure in the ancestral species nor homonomous to any other structure in the same organism" (Müller and Wagner 1991, p. 243). However, over the past three decades, the evo-devo revolution thoroughly reworked the concept of homology away from a binary concept (two structures either are or are not homologous) and toward what essentially amounts to a *layered gradient*: structures can share homology on some level of biological organization but not others, and even what they share can be *partial*. As such, evo-devo re-conceptualized the relationships between homology and innovation and diversification in ways that fully aligned with the one concept that founded evolutionary biology over 150 years ago – *descent with modification* (Wake, Hall, and Olson 2006; Moczek 2008). It also put researchers in a position to stop debating what does and does not qualify as a novelty (though of course some still do) and focus more on the process of innovation in developmental evolution and the rules that govern *when* and *how* increasingly divergent phenotypes may be allowed to rise from within the confines of ancestral variation.

Our understanding of Hox genes also underwent significant transformations over recent decades. Specifically, increasing knowledge about when, how, and where in development Hox genes execute their functions resulted in a shift away from the notion of *master regulators*, i.e., genes acting as a switch turning on or off a gene network module and its resulting developmental process, and toward the concept of *conditional regional specifiers*, in which the presence of specific transcription factors conveys spatial information and enables region-specific regulation of downstream effectors of developmental processes. This became even more evident when experiments showed that Hox proteins need to be present throughout ontogeny to complete developmental trajectories, rather than only at their onset (Weatherbee et al. 1998);

in other words, Hox genes seem to be acting more like persistent micromanagers than master genes (Akam 1998).

These views were reinforced by the many examples of novel traits and functions emerging from gene cooption, neofunctionalization, or gene network rewiring. These scenarios all share that they necessitate the ancestral expression of co-opted genes at the same developmental time or location, or both, as well as the existence of mechanisms that permit genes to be recruited into novel contexts *without* interfering with their ancestral functions (McQueen and Rebeiz 2020). One such mechanism, discussed in more detail below, is *latency* of expression, i.e., gene expression lingering on beyond the precise developmental stages and spatial domains in which it is ancestrally required (Rebeiz et al. 2011). Thus, genes contributing to successful cooption events may be those that, rather than being subject to a tightly choreographed turning on (or upregulation) in precise locations, are more promiscuously expressed (in space or time, or both) than strictly necessary to support their ancestral roles. Hox genes, whose layered pattern of antero-posterior functions results from the successive inhibition of anteriorly expressed genes by more posteriorly expressed ones (Duboule and Morata 1994), fit this scenario quite well.

The subsequent development of new techniques such as high throughput sequencing, RNA interference (RNAi), and now CRISPR-Cas9, alongside the establishment of diverse new model systems, further revolutionized the field. Researchers are now in a position to apply these perspectives well beyond Hox genes and to probe the existence of *common themes*, such as the role of gene latency in cooption events during the genesis of novelty in animal developmental evolution. In this chapter, we will highlight one such new model system, horned beetles in or closely related to the genus *Onthophagus*, and what we have learned (through the study of Hox genes and other transcription factors) regarding the nature of what we call the *innovation gradient:* starting with the baby steps of developmental evolution, followed by subsequent elaboration, eventually yielding structures that at least on the surface strike us as entirely novel. We begin with a brief primer on the biology of horned beetles.

3.2 A PRIMER ON BEETLE HORNS AND HORNED BEETLES

Horns or horn-like structures can be observed in diverse beetle families, with the majority of species, morphological diversity, and degree of exaggeration being concentrated in two subfamilies within the Scarabaeidae, the Dynastinae (rhinoceros beetles), and Scarabaeinae (true dung beetles) (rev. in Snell-Rood and Moczek 2013). In the vast majority of species, horns extend from either the dorsal head or the dorsal first thoracic segment, or both. As such, beetle horns are not modified legs, mouthparts, or antennae and instead form in body regions normally not tasked with producing appendages or any other type of outgrowth (Moczek and Nagy 2005). Consequently, beetle horns were generally viewed as lacking obvious homology to other structures in insects or non-insect arthropods, a notion we will challenge below, at least for thoracic horns.

Beetle horns are also well known for their extraordinary diversity among and within species (Figure 3.1; Moczek 2005). Among species, even closely related

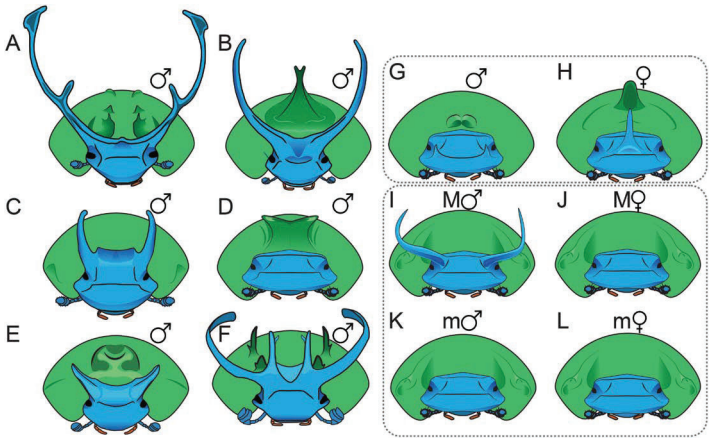

FIGURE 3.1 Onthophagine beetles boast tremendous diversity in the number, shape, and partly location of both thoracic (green) and cephalic (blue) horns at both interspecific and intraspecific (sex- or morph-specific) levels. (A) *Onthophagus rangifer*; (B) *Onthophagus atripennis*; (C) *Onthophagus australis*; (D) *Onthophagus binodis*; (E) *Onthophagus watanabei*; (F) *Onthophagus multicornis*; (G–H) *Onthophagus sagittarius* male (G) and female (H) morphs; I–L) *Onthophagus taurus* major male (I), major female (J), minor male (K) and minor female (L) morphs.

species within a single genus, horns may differ widely in relative size, location, number, and shape (Figure 3.1A–F). This interspecific variation is a reflection of evolved, developmentally canalized differences between species. However, much diversity also exists *within* species (Figure 3.1G–L), predominantly on two levels: first, horns are commonly restricted to, or greatly elaborated in, males compared to females, a consequence of sex-specific development following XX/XY sex determination common in many beetle families (Kijimoto et al. 2013). Second, males are frequently variable in horn development themselves, in many cases resulting in the formation of relatively discrete horned or hornless, or *major* and *minor*, morphs. In the latter category, male dimorphisms can sometimes be so extreme and discrete that the resulting morphs have initially been described as belonging to separate species (Paulian 1935). Variation in horn growth among conspecific males is primarily a consequence of nutritional variation experienced during the larval stage: male larvae with access to optimal feeding conditions grow to body sizes that permit metamorphosis into large male adults, which exhibit fully formed horns, whereas male larvae with access to suboptimal feeding conditions metamorphose into smaller male adults, often with greatly reduced degrees of horn development (Moczek and Emlen 1999). The resulting variation in horn growth in males is thus closely tied to adult body size and larval nutrition, in contrast to females in which reduced or absent horn growth is a reflection of canalized, sex-specific development regardless of larval nutrition and adult female size. This extraordinary diversity of horn phenotypes on different levels of biological organization (among species, sexes, individuals) due to different proximate reasons has motivated much research into the mechanisms that facilitate species-, sex-, and nutrition-specific development, its interactions, and its evolution

(Wasik and Moczek 2011, 2012; Wasik, Rose, and Moczek 2010; Kijimoto, Andrews, and Moczek 2010; Kijimoto, Moczek, and Andrews 2012; Casasa and Moczek 2018; Casasa, Zattara, and Moczek 2020).

The tremendous diversity in horn phenotypes notwithstanding, all horns – when present – appear to be used for the same purpose: aggressive male combat over access to females directly, or breeding opportunities females depend on, such as nesting sites and burrows. In each species studied so far, "males were found to use their horns to push, block, prod, stab, lift, dislodge or otherwise impede rival males from accessing females" (Kijimoto et al. 2013). Such fights are often intense, energetically expensive, time consuming, but rarely cause injury (Snell-Rood and Moczek 2013). Horns are effective, and large-horned males enjoy an advantage in fights, whereas small males, by virtue of their body size, generally fail to succeed in fights against larger rivals (Moczek and Emlen 2000). However, in many taxa such males have instead specialized to employ alternate reproductive "sneaker" tactics: for example, minor males invest heavily into testes development and sperm competition, and utilize non-aggressive behaviors to circumvent physically superior rival males (Moczek and Emlen 2000; Simmons, Emlen, and Tomkins 2007). In these males, the absence of horns may not just reflect the lack of utility for a weapon, but has also been shown to improve agility of small sneaker males (Moczek and Emlen 2000; Madewell and Moczek 2006). Taken together, fully horned, large, fighter males on one side, and largely hornless, smaller, sneaker males on the other, thus reflect alternative morphological and behavioral adaptations to competition over a limited resource – females. Note, however, that horned and hornless males do *not* reflect different genotypes: instead, they embody alternate, *polyphenic*, morphs, expressed in a context-dependent manner, much like castes in social insects or seasonal morphs in butterflies. Nor do small hornless males reflect starvation phenotypes. Instead, past work has shown that hornlessness in these males does not result from the *inability* to make horns, but from the *active repression* of horn formation (via *Hedgehog* signaling; Kijimoto and Moczek 2016). In the sections that follow, we first introduce what is currently known about the Hox gene cluster in *Onthophagus* horned beetles, and then focus on the developmental mechanisms that induce horns in the first place, regardless of whether they are elaborated or reduced, form in a male or female, or represent singular, paired, or otherwise formed structures. The only distinction that will emerge as absolutely critical is one of location: we will try to convince the reader that the diversity of head and thoracic horns may be underlain by many of the same developmental mechanisms, yet that their origin itself is not.

3.3 HOX GENES IN HORNED BEETLES

Current knowledge of Hox gene genomic organization and developmental function within Coleoptera comes mostly from studies in the flour beetle *Tribolium castaneum*. In this species, Hox genes are tightly linked into a single cluster spanning approximately 756 Kb and contain all eight members originally described in *Drosophila* fruit flies – *labial (lab), proboscipedia/maxillipedia (pb/mxp), Deformed (Dfd), Sex combs reduced/Cephalothorax (Scr/Cx), Antennapedia/prothoraxless (Antp/ptl), Ultrabithorax/Ultrathorax (Ubx/Utx), abdominal A (abdA),* and *Abdominal B (AbdB)* – plus *fushi tarazu* (*ftz*) and *zerknült* (*zen*) (Figure 3.2A; Shippy et al. 2008). Functional evidence so far suggests Hox genes play similar roles

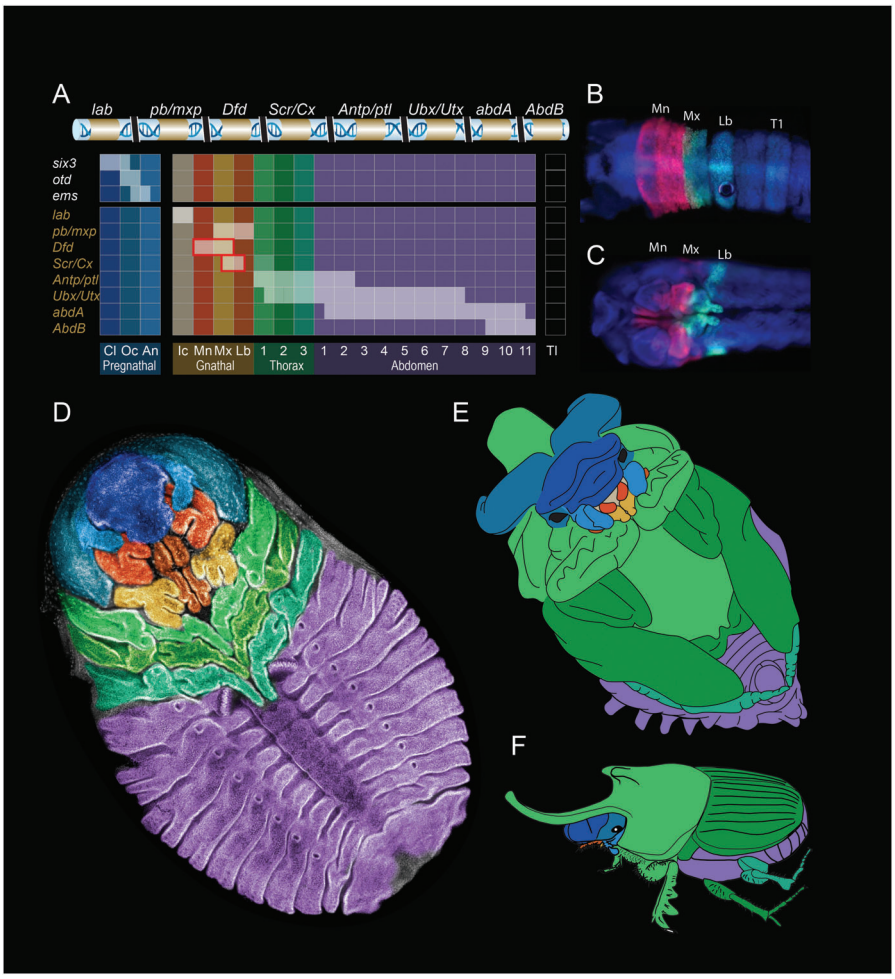

FIGURE 3.2 Hox genes in beetles. (A) All eight canonical Hox genes first described in *Drosophila* have been found in the genomes of the flour beetle *Tribolium castaneum* and of the horned beetle *Onthophagus taurus*. Expression data along the segmented embryo for all genes are available only in *Tribolium* (lighter boxes), however, preliminary data for *Ot-Dfd* and *Ot-Scr* (red-framed boxes) show similar patterns. (B)–(C) *In situ* expression assays for *Ot-Dfd* (red) and *Ot-Scr* (turquoise) in early embryos of *O. taurus* at two different stages. (D) Ventral view of a mid-stage embryo of *O. taurus*, false colored to highlight the different segmental identities (see key in A). (E)–(F) Pupa of a male *O. taurus* (E) and adult of a male *O. nigriventris* (F), with structures color coded to show approximate correspondence with embryonic segments in D. Expression data in (A) based on Brown et al. (2002); (B)–(C) based on D. Linz & A. Moczek, unpublished data. Segment abbreviations: Cl – clypeolabral; Oc – ocular; An – Antennal; Ic – Intercalary; Mn– mandibular; Mx – maxillary; Lb – labial; T1 – first thoracic; Tl – telson.

in beetle and fly embryonic development, and that at least some of those roles (e.g., specifying appendage anteroposterior identities) are also important during post-embryonic development (Smith and Jockusch 2014). Among horned beetles of the genus *Onthophagus*, only the genome of the bull-headed dung beetle *Onthophagus taurus* is currently available (Zattara et al. 2016c; Thomas et al. 2020). Through a combination of automatic (Zattara et al. 2016b) and manual annotation, single-copy orthologues of all eight fly genes plus *ftz* and *zen* have been identified; however, the assembly quality of the current draft is too low to adequately assess the size and degree of conservation of the Hox cluster itself, and re-sequencing of this genome (and *de novo* sequencing of two additional onthophagine genomes) is currently in progress. Although expression of Hox genes during embryonic development has only been described for *Dfd* and *Scr*, the patterns at least for these two genes are similar to those found in *Tribolium* (Figure 3.2B–D). Given their well-known role in specifying the identity of many structures along the body axis, it would be reasonable to expect that Hox genes are playing a corresponding role in the formation of horned beetles during post-embryonic development (Figure 3.2D–F). In the following sections, we show how recent assessments of the role of Hox and other embryonic patterning transcription factors in *Onthophagus* beetles has provided surprising insights into the developmental and evolutionary origins of thoracic and head horns.

3.4 THE ORIGINS OF THORACIC HORNS

The term *thoracic horn(s)* is a shortcut to refer to projections emanating from the pronotum, the dorsal most thoracic sclerite of the first thoracic, aka *prothoracic* segment, also often abbreviated as T1. This prothoracic segment, in contrast to the second (T2, mesothoracic) and third (T3, metathoracic) segment, never bears wings in extant insects, though fossil evidence shows that in some early lineages it supported the formation of at least wing-like structures (Grimaldi and Engel 2005). However, present-day insects actively suppress the formation of such structures on T1, an inhibitory function executed in all insects studied to date by the prothorax-specific Hox gene *Sex-combs reduced* (*Scr)* (Struhl 1982; Hughes and Kaufman 2000; Curtis et al. 2001; Tomoyasu, Wheeler, and Denell 2005). Interestingly, the insect prothorax has emerged as a remarkable hotspot for innovation across insect orders (Figure 3.3). Apart from the formation of prothoracic horns in beetles, innovations include the prothoracic, often leaf-like enlargements in many grasshoppers (e.g., Tetrigidae), the extraordinarily diverse "helmets" of treehoppers (Membracidae), and pattern formation, such as the eye spots of click beetles (Elateridae) enabled through the two-dimensional arrangement of colored scales, akin to butterflies. To probe the very origins of these T1-specific innovations, recent work has focused on the role of the gene network underpinning the formation of insect wings, themselves perhaps one of the most enigmatic of innovations in animal evolution. Before we review the main insights gained from this approach, it will be helpful for us to take a brief detour and summarize recent developments in our understanding of the origins of insect wings.

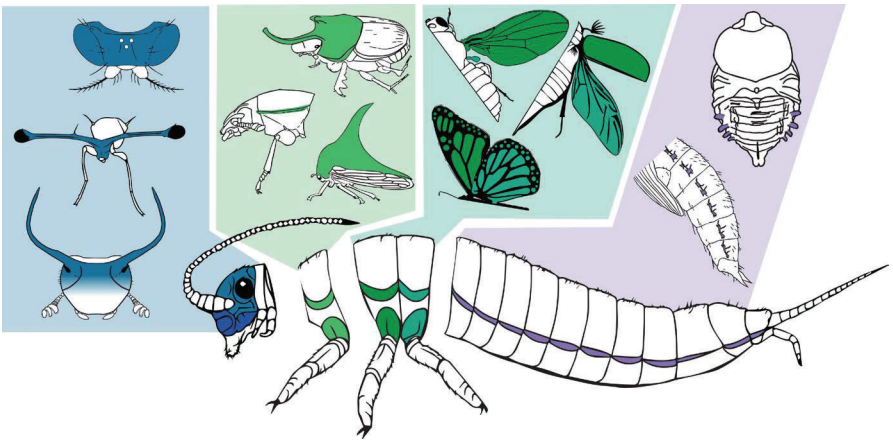

FIGURE 3.3 Hotspots of innovation within the insect body plan. Four regions from a hypothetical ancestral apterygote insect from which a diversity of morphological novelties has emerged: the anterior, presumptively Hox-free dorsal head (blue) that evolved into the reduced head capsule of cyclorraphan flies, the eye stalks of stalked-eye flies or the cephalic horns of scarab beetles; the first thoracic segment T1 (leftmost green) that evolved structures ranging from the modest marginal carinae of tenebrionids to the spectacular prothoracic horns of scarab beetles or the helmets of tree-hoppers; the second and third thoracic segments (T2 and T3) in which pterygote wings evolved and were further elaborated into, for example, halteres and elytra; or the lateral margins of the abdominal segments (purple) where functionally important structures like gin traps or pupal support structures have originated.

3.4.1 Wings and Wing Serial Homologs

The origin of insect wings has fueled a century-long debate, with two hypotheses polarizing the discussion. Accordingly, insect wings arose as expansions of the notum, the dorsal plate of thoracic segments (Hamilton 1971; Rasnitsyn 1981), or alternatively, from the exite of an ancestral additional proximal leg segment present prior to the origin of insects and since absorbed into the (pleural) side wall of segments (Kukalová-Peck 1983; Averof and Cohen 1997). These competing *notal expansion* and *exite* hypotheses recently became united in the *dual origin* hypothesis which posits that wings are composite structures (Figure 3.3, green), contributed to by both notal and pleural sources, and hence two distinct *wing serial homologs* (Clark-Hachtel, Linz, and Tomoyasu 2013). Studies on a diversity of insects (Niwa et al. 2010; Medved et al. 2015; Elias-Neto and Belles 2016; Prokop et al. 2017; Clark-Hachtel and Tomoyasu 2020), including paleontological, morphological, gene expression, and gene function approaches, collectively now provide strong support for this hypothesis. Further work in *Tribolium* (Linz and Tomoyasu 2018; Hu et al. 2018) and *Tenebrio* (Ohde, Yaginuma, and Niimi 2013) beetles also shows that homologous source tissues and resulting morphological structures are not restricted to currently wing bearing (T2, T3) segments but instead can also be found in the prothoracic segment (T1) as well as all abdominal segments: for instance, structures

known as gin traps which can be found laterally on each abdominal segment during the pupal stage (Figure 3.3, purple) are now understood to constitute partial wing serial homologs. We will return to specifically focus on these structures later in this chapter.

Collectively, these findings support the notion that the presence of two distinct sets of wing serial homologs per segment may reflect the ancestral condition of thoracic *and* abdominal segments, that the existence of these wing serial homologs *predate* the actual origin of wings, and that at least some insect lineages succeeded in utilizing wing serial homologs to evolve structures other than wings outside T2 and T3 (Linz and Tomoyasu 2018; Hu et al. 2018; Ohde, Yaginuma, and Niimi 2013; Hu and Moczek 2021). A related argument was put forth years earlier to explain the origin of the helmet of treehoppers, hemipteran insects famous for the highly diversified elaborations of the prothorax (Figure 3.3). In a landmark study, Prud'homme et al. (2011) posited that a de-repression of the T1 wing homolog network through unknown mechanisms enabled its re-use in the context of helmet evolution. However, subsequent work (Mikó et al. 2012; Yoshizawa 2012) challenged the validity of the data presented (anatomical and gene expression data, but no functional analyses), and to date the homology status of the treehopper helmet remains heavily debated (e.g., Fisher et al. 2020). In contrast, the work on *Tribolium* and *Tenebrio* highlighted above yielded results that fully meet the standards needed to establish wing serial homology: (i) functional analysis of cardinal wing genes documenting their involvement in a hypothesized wing serial homolog, as well as (ii) transformation of putative wing serial homologs into structures with unambiguous morphological wing identity. These same standards were recently applied to understand the origins of prothoracic beetle horns.

3.4.2 On Horns and Wings

Prothoracic horns form outside wing bearing thoracic segments, and superficially bear no resemblance to wings: horns are heavily sclerotized, non-hinged outgrowths, and in many instances singular, and medially positioned, in contrast to the paired and bilaterally positioned wings found on T2 and T3. Nevertheless, several sets of evidence now provide strong support for the hypothesis that thoracic horns on T1 and *bona fide* wings on T2 and T3 are at least partially serially homologous. First, diverse genes required for the formation and proper patterning of wings are also functionally required for the formation of thoracic horns in three horned beetle species: these include the wing selector gene *vestigial*, genes critical to the early patterning of wing formation (*apterous, homothorax, decapentaplegic, abrupt*), and key members of the *hedgehog* (*patched, cubitus interruptus*) and *wingless* signaling pathways (*pangolin, disheveled*) (Hu, Linz, and Moczek 2019; Moczek and Rose 2009; Wasik and Moczek 2011; Wasik and Moczek 2012; Kijimoto and Moczek 2016). Second, hypomorphic downregulation of a subset of these target genes resulted in the retention of paired, bilateral vestiges of the thoracic horn similar to what can also be observed in wildtype individuals late in larval development, suggesting that even single horns medially positioned in the adult may derive from paired, bilateral source tissues (Hu, Linz, and Moczek 2019).

Perhaps the most critical evidence came from functional analyses of the T1-specific Hox gene *Sex combs reduced (Scr).* In line with results from a diversity of studies in other taxa (Struhl 1982; Hughes and Kaufman 2000; Curtis et al. 2001), RNAi-mediated transcript depletion of *Scr* induces large, ectopic T1 wings, and, as in *Tribolium,* these ectopic T1 wings take on the identity of forewings, or elytra (Clark-Hachtel, Linz, and Tomoyasu 2013). However, in horned beetles this induction is paralleled by a loss or reduction of the prothoracic horn, consistent with the hypothesis that in wild-type individuals, *Scr* may be mediating a transformation of bilateral T1 wing homologs into dorso-medial prothoracic horn tissue (Hu, Linz, and Moczek 2019). Moreover, analysis of hypomorphic *Scr*RNAi phenotypes shows an inverse relationship between prothoracic horn tissue retained and size of the ectopic wing induced. Lastly, joint functional perturbation of *pannier (pnr)* and *Scr* established a correspondence between thoracic horn tissue in wildtype individuals and ectopic T1 wing tissue in *Scr*RNAi animals: by itself, *pnr*RNAi removes dorsomedial projections, including the thoracic horn, yet does not affect the formation of T2 or T3 wings. As such, it provides an experimental opportunity to genetically ablate thoracic horn tissue *without* impacting wing formation. Ectopic T1 wings formed as a consequence of *Scr* knockdown alone are prominent, large structures, yet ectopic wings formed in a *pnr*RNAi background are consistently and measurably smaller, and further lack the dorsal surface traits observed in *Scr* single-knockdown individuals (Hu, Linz, and Moczek 2019). Collectively, these results support that prothoracic horn primordia contribute to ectopic, bilateral T1 wings, and by extension, that thoracic horns, ectopic wings, and regular fore and hind wings are all at least partially serially homologous to each other.

More generally, these findings may help explain why the insect prothorax has emerged as a hotspot of evolutionary innovation: as now demonstrated for beetle thoracic horns, and hypothesized for many other insect groups, prothoracic wing serial homologs under the control of *Scr* may serve as critical substrate toward the formation of diverse novel features in this body region (Figure 3.3). If correct, the evolution of the gene network governed by *Scr* must be playing a critical role in this process. For example, the same study that assessed the function of diverse wing-related genes in thoracic horn formation (Hu, Linz, and Moczek 2019) also executed a comparative RNAseq study of wings, as well as known or presumed wing serial homologs, and tissues unrelated to wing formation. While all wing-related tissues were found to share the expression of *vestigial*, expanding this approach to 41 genes known to be functionally required for some aspect of wing formation showed a significant transcriptional divergence of thoracic horn tissue from other wing serial homologs. Furthermore, transcriptome-wide clustering of all 4191 differentially expressed transcripts revealed no clustering pattern corresponding to wing relatedness among any of the tissues examined. This result suggests that wing serial homologs such as those existing in T1 may serve as developmental-genetic *starting points*, followed later in development by the establishment of structure-specific transcriptional repertoires, in turn facilitating morphological differentiation events specific to each trait. If correct, this suggests that the downstream repertoire of *Scr* must exhibit remarkable evolutionary lability, able to support the independent evolution of highly diversified outgrowths in diverse hemi- and holometabolous orders. In the next section, we explore

how a similar interplay between Hox genes, wing serial homologs, or other target genes may also contribute to morphological diversification and innovation outside the thorax.

3.4.3 Hox Gene–Mediated Innovation Outside the Thorax

Work on the beetle genera *Tribolium* (Linz and Tomoyasu 2018; Hu et al. 2018) and *Tenebrio* (Ohde, Yaginuma, and Niimi 2013) showed that wing serial homologs and corresponding morphological structures need not be restricted to thoracic segments but instead can also be found in all the abdominal segments: for instance, *gin traps,* which can be found bilaterally on each abdominal segment during the pupal stages of *Tribolium* and *Tenebrio*, consist of bifurcated, strongly sclerotized outgrowths with toothed flanges, and are used as defensive structures (Hinton 1946; Wilson 1971; Eisner and Eisner 1992). Work by Hu et al. (2018) showed that most of the upstream, but not downstream genes, within the hierarchy of the wing gene regulatory network are indeed required for the correct formation of gin traps, and established that much like thoracic horns in *Onthophagus*, gin traps in both *Tribolium* and *Tenebrio* share partial serial homology with other wing-related tissues (Ohde, Yaginuma, and Niimi 2013). Intriguingly, while abdominal appendages and projections are restricted to the posterior-most abdomen in most adult insects (e.g., genitalia, ovipositors, cerci), such outgrowths are common throughout abdominal segments in immature stages: a great diversity of pupae, nymphs, and larvae possess bilateral and segmentally reiterated spikes, blades, or gin trap-like outgrowths (Figure 3.3, purple). This raises the question whether the extraordinary diversity found in these abdominal projections might similarly be enabled through the reuse and subsequent diversification of wing serial homologs.

The first additional data able to put this intriguing hypothesis to the test are now available from horned beetles. Ignoring the possession of horns for the moment, the subfamily Scarabaeinae, or true dung beetles, to which a large fraction of horned beetles belong, is also famous for the formation of pupae with bilateral and segmentally reiterated projections. These so-called *pupal support structures* are taxonomically widespread and have been proposed to facilitate the correct positioning of pupae in underground pupation chambers and distancing from substrate to minimize microbial infections (though experimental testing of these hypotheses is still pending). A first functional genetic analysis of these structures (Hu and Moczek 2021) found that out of 14 genes prominently positioned within the wing gene regulatory network, at least 10 were functionally required for the proper formation of 2 types of pupal support structures; 8 of those genes were also required for the proper formation of thoracic horns. Taken together, these data support the hypothesis that pupal support structures also constitute partial wing serial homologs, as might the extraordinary diversity of abdominal outgrowths so common in arthropods. Here the functional significance of abdominal Hox genes such as *abdA* or *AbdB* in establishing structure-specific transcriptional landscape *and* their lineage-specific diversification is largely unknown and constitutes a promising avenue for future research. Yet at least some functional perturbations already hint at intriguing interactions: for example, in bioluminescent *Photuris* fireflies, downregulation of *AbdB* not only results in

a spectacular homeotic transformation of genitalia to legs, but also a severe disruption of lantern formation in the 6th and 7th abdominal segments, including a deletion of the bioluminescent lantern and a loss of transparent cuticle that normally overlays this organ (Stansbury and Moczek 2014).

3.5 THE DEVELOPMENTAL EVOLUTION OF HEAD HORNS: PARALLEL INNOVATION IN A HOX-FREE SPACE?

Horned beetles are also well known for a great diversity of horns located on the dorsal surface of the head, usually referred to as *head (or cephalic) horns*. Recall that like their thoracic counterparts, head horns are elongated, often pointed, non-articulated extensions of the body wall, and show a diversity of shapes and nutrition-responsiveness across species (Figure 3.1). In fact, many species sport both thoracic *and* head horns which often function in a tightly integrated manner as pincers or clamps able to grab, secure, lift, and throw opponents during combat (reviewed in Hu et al. 2020; Moczek 2005). All of this would suggest both horn types are likely to be serial homologs, or at least to be regulated by a common regulatory gene network. However, current evidence not only fails to support that thoracic and cephalic horns are serial homologs, or homologs of any kind, but instead points at their completely independent evolution through their reliance on discrete gene regulatory networks, one operating within a Hox expression region governed by *Scr* and the other within a presumptively Hox-free space.

3.5.1 Head Horn Development and Diversity

Like thoracic horns (see above), head horns form as folded outgrowths of the developing pupal epidermis during the prepupal stage, underneath the head capsule of the last larval instar. After shedding the larval head capsule at pupation, head horns unfold and inflate as the internal hemolymph pressure pushes them outward (Moczek, Cruickshank, and Shelby 2006; Gotoh et al. 2021); during the pupal stage, pupal horns are further sculpted into their final, adult morphology (Moczek 2006, Kijimoto et al. 2010).

The extraordinary diversity of head horn shapes found within the Onthophagini can nonetheless be categorized into two main types of horns: far more common posteriorly placed horns (as seen, for example, in *Digitonthophagus gazella*, *O. taurus*) and much rarer anterior horns (as seen in male *O. sagittarius*) (Figure 3.1) (Emlen et al. 2005). Cell fate mapping experiments using ablation of epidermal tissue from the dorsal larval head established that despite their large morphological diversity, posterior horns derive from tissue located along the boundary between two embryonic head "segments", the posterior ocular and the anterior clypeolabral regions (Busey, Zattara, and Moczek 2016). Anterior horns, in contrast, derive from larval head regions fully contained within the clypeolabral region. Studies in *O. taurus*, a species with male-specific posterior horns, have shown that at the prepupal stage, when future horns first develop, anterior and posterior head regions exhibit marked differences in their transcriptional landscapes: the anterior region presents a more homogeneous landscape with little medial-to-lateral differentiation, while

the posterior region presents a much more heterogeneous profile and more marked medial-to-lateral differentiation (Linz and Moczek 2020). Intriguingly, these differences are observed in both horn-bearing males and hornless females, suggesting that it may reflect a general property of the dorsal beetle head irrespective of the presence or absence of horns.

3.5.2 The Dorsal Head: A Hox-Free Space?

Given that anterior Hox genes are important for proper specification of several structures in the arthropod head (e.g., mouthparts), they were likely candidates for a role in the spectacular elaborations of cephalic structures found in many insect species in general (Figure 3.3, blue), and specifically for a role in head horn development. However, most experiments to date have consistently failed to produce evidence for their involvement in instructing head horns; in fact, the dorsal region of the head of most insects has proven surprisingly oblivious to Hox gene manipulations, even when corresponding ventral regions had shown a phenotype. Such lack of response of dorsal head tissues to Hox manipulation stands in stark contrast to other parts of the body, in which both ventral and dorsal regions are affected by homeotic transformations (e.g., the transformation of halteres to wings *and* T3 to T2 legs in *Drosophila Ubx* mutants; Weatherbee et al. 1998) and strongly suggests that the assembly of the insect dorsal head differs fundamentally from that of the rest of the body.

Currently, the best explanation for these differences is the "bend-and-zipper" model of head development, which posits that during insect embryonic development, the anterior-most region of the embryo – the future clypeolabral region – bends dorsally, then is bilaterally overtaken by the ocular region, followed by the medial fusion ("zipping") of the left and right ocular region into one continuous ocular "segment" (Figure 3.4A; Posnien et al. 2010). Evidence for this model comes primarily from detailed studies of "anterior" gene expression during embryonic development in the flour beetle *T. castaneum* (Schinko et al. 2008; Posnien, Bashasab, and Bucher 2009; Posnien 2009; Posnien and Bucher 2010; Posnien et al. 2011). Importantly, the anterior-most region of the body of most bilaterians, including arthropods, is ancestrally free of Hox gene expression and is instead patterned by an independent and unique gene network, which includes *optix/six3* and *orthodenticle* (*otd*), two transcription factor-encoding genes exhibiting complementary expression domains at the clypeolabral-ocular boundary (Figure 3.4B; Li et al. 1996; Posnien et al. 2011). Both genes have been shown to be ancestrally expressed at the anterior end of bilaterians (Steinmetz et al. 2010), including beetles; in insects, the process envisioned by the "bend-and-zipper" model introduced above therefore results in a head with a *ventral* compartment formed by Hox expressing regions, yet a seemingly complementary dorsal compartment comprised by a Hox-free space; i.e., a head in which dorsal and ventral tissues located in the same antero-posterior position have originated from sources located at initially different, even non-adjacent, antero-posterior positions in the early embryo. This model explains the resilience of the dorsal head to Hox gene manipulations and implies that structures evolving within the dorsal head domain should not be able to link to gene regulatory networks that depend on Hox gene expression. In other words, dorsal head structures – like head horns – cannot

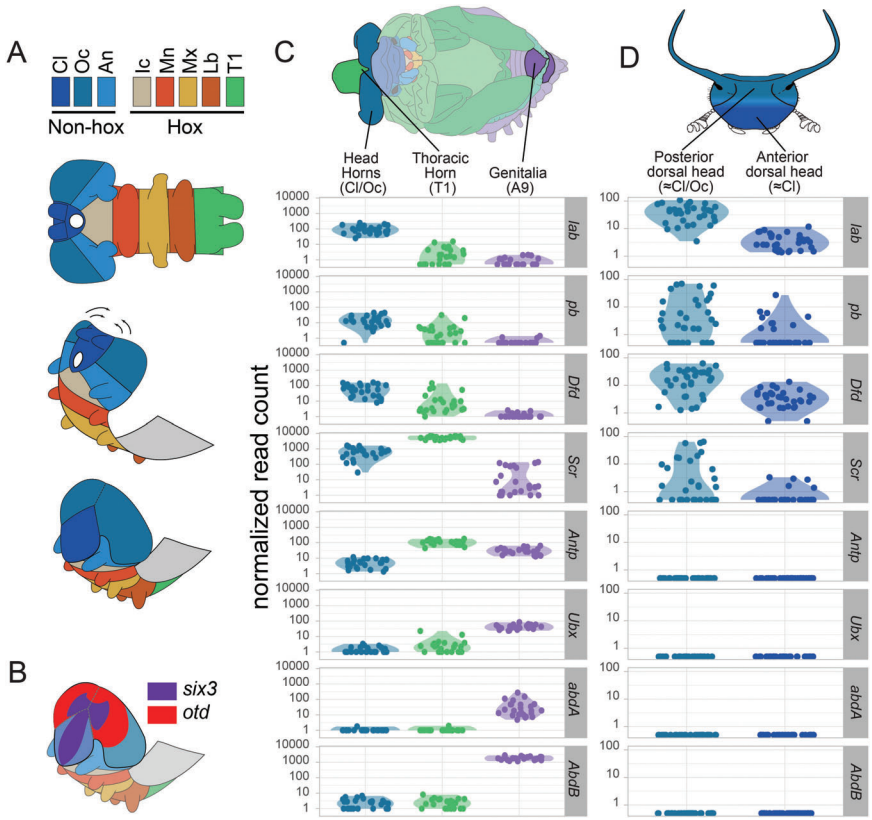

FIGURE 3.4 (A) The *bend and zipper* model of head formation (redrawn after Posnien et al 2011). (B) Patterns of embryonic expression of *six3* (violet) and *orthodenticle* (*otd*, red) in the embryonic dorsal head after dorsal closure. (C) Expression of Hox genes at the early pupal dorsal head epithelium (CHE), dorsal T1 epithelium (THE), and abdominal genitalia (GEN). (D) Expression of Hox genes at the anterior (A) and posterior (P) compartments of the prepupal dorsal head. RNAseq data used in (C) from Ledón-Rettig, Zattara, and Moczek (2017); RNAseq data used in (D) from Linz and Moczek (2020).

be expected to be serially homologous to structures developing from Hox regulated body regions – like thoracic horns.

This is confirmed by experimental observations: experimental knockdown of regulatory members of the gene networks directing the development of wings, thoracic horns and their serial homologs do not affect head horn development (Hu, Linz, and Moczek 2019). In contrast, head horn development is disrupted after interference with any of several genes ancestrally tasked with instructing embryonic head formation (Zattara et al. 2016a; Zattara et al. 2017; Linz and Moczek 2020). For example, postembryonic knockdown of *otd* drastically alters adult dorsal head patterning including the formation and positioning of horn development, causes severe reduction of normal posterior head horns, and even more strikingly, induces the formation

of additional ectopic horns in the anterior region of the dorsal head (Zattara et al. 2016a). Importantly, the effects of *otd* knockdown on head horns do not appear to be a collateral result of broader effects on beetle head development, since the same manipulations have no effect in the head of *Tribolium* (however, RNAi phenotypes *outside* the head are shared in both taxa). Additionally, two independent studies implicate components of the gene network underlying eye patterning: Linz and Moczek (2020) documented sex-specific expression of eye patterning genes in posterior head compartments (note, actual eye forming regions were removed during sample preparation) and Zattara et al. (2017) demonstrated that *otd*RNAi in *Onthophagus* (but not *Tribolium*) induces the formation of *functional* ectopic compound eyes in the posteromedial dorsal head. More generally, these findings demonstrate that head horns are not serial homologs of thoracic horns, wings, or any other segmentally reiterated structure, and thus neither rely on wing nor Hox genes in their development. Instead, head horns emerge as patterned at least in part by genes ancestrally tasked with instructing the formation of the embryonic head, and possibly genes otherwise well-studied for their role in the formation of compound eyes and ocelli. Intriguingly, while ocelli (typically three single-lens eyes located on the posterior medial head of most insect orders) are positioned similar to posterior head horns, they constitute a structure secondarily lost in the early stages of beetle evolution (Leschen and Beutel 2004).

But, is the dorsal head truly and entirely a Hox-free space? In contrast to *Tribolium*, there are very few data that would permit an assessment of embryonic Hox gene expression in *Onthophagus*, yet preliminary work suggests congruence between anterior Hox gene expression between *Onthophagus* and what has been described for flour beetles and flies (Figure 3.2B–C; D. Linz and A. Moczek, unpublished data), at least for embryos. Available expression data on postembryonic development come mainly from RNAseq experiments, and here things become more complicated. In a study in which four tissues located at different positions along the body were sampled at an early pupal stage (Ledón-Rettig, Zattara, and Moczek 2017), transcription of all eight *O. taurus* Hox genes showed a distribution pattern similar to that described for many insect embryos (Figure 3.4C). Notably, anterior Hox genes not only remain transcriptionally active as late as metamorphosis, but they seem to be expressed *even in the dorsal head*. Additional confirmation comes from the genome-wide study of the transcriptional landscape of prepupal dorsal head regions (Linz and Moczek 2020) highlighted already, showing that several Hox genes seem to have expanded their expression beyond their presumed gnathal domains (Figure 3.4D): *labial*, *proboscipedia*, and *deformed* exhibit compartment-specific expression in the dorsal head in clear contradiction to *Tribolium*'s bend and zipper model and findings in diverse other taxa (Posnien and Bucher 2010), making the dorsal *Onthophagus* head a *not*-so-Hox-free space. Functional analyses are currently ongoing.

3.5.3 Developmental Inertia and Gene Latency

To date, all evidence points to a separate and independent origin of head and thoracic horns in scarab beetles, even though both horn types do share at least some patterning genes (e.g., the proximodistal polarity genes *homothorax* and *Distal-less*;

Moczek and Nagy 2005). Similarly, *otd*RNAi affects both head horns and pupal/adult thoracic horns (Zattara et al. 2016a), though the latter is likely a consequence of *otd*'s general and evolutionarily conserved function in patterning dorsal and ventral midline structures along the body axis. Nevertheless, had this gene been the first or only gene to be investigated, it would have likely prompted the conclusion that both types of horns were serial homologs. Thus, claims of serial or even "deep" homology stemming from finding a common role for one or few genes should be approached with caution.

Although *otd* has a critical role during embryonic head formation in (ancestrally hornless) *Tribolium* beetles (Schröder 2003; Kotkamp, Klingler, and Schoppmeier 2010), *otd* seems to play no role in their post-embryonic head development, in stark contrast to *Onthophagus* beetles. Interestingly, *otd* is expressed in both *Tribolium* and *Onthophagus* heads at larval, prepupal, pupal, and adult stages (Zattara et al. 2016a). While a different, yet to be described role for *otd* in postembryonic heads of *Tribolium* cannot be ruled out, it is tempting to speculate that through developmental inertia (defined as a tendency for expression of a given gene to remain *on* past the developmental stages in which it had a function), genes such as *otd* (and possibly the Hox genes discussed above) may retain latent, non-adaptive, yet tissue-specific expression in the dorsal head of beetles. Additional evidence for such a scenario derives from the transcriptional assessment of dorsal head compartments by Linz and Moczek (2020) in *O. taurus*: a subsequent functional analysis of 22 genes showing robust localized expression in the dorsal head, including 9 genes well known for their role in embryonic head patterning, failed to document a functional role in head and/or horn formation, despite unambiguous knockdown phenotypes elsewhere in the body. A study using the same approach in a distantly related horned species, the rhinoceros beetle *Trypoxylus dichotomus*, reported a similar result: a majority of genes found to be differentially expressed in horn-producing tissues and tested for function failed to show evidence of a role in horn development (Ohde et al. 2018). This pattern, however, may be evolutionarily labile: despite lacking a role in horn development in *O. taurus*, knockdown of three genes (*retinal homeobox*, *cap'n'collar*, and *Sp8*) in the closely related *O. sagittarius* strongly reduced the size of the anterior horns found in males of this species (Linz and Moczek 2020). Taken together, current data thus strongly support a scenario whereby horn development evolves by dynamically adding and removing gene network members that exhibit latent, non-adaptive, tissue-specific expression as a result of developmental inertia.

3.6 WHAT HAVE WE LEARNED, AND WHERE DO WE GO FROM HERE?

The study of horned beetles has further confirmed emerging themes in the study of innovation in developmental evolution, as well as expanded our perspectives in directions that we hope will motivate future research. For instance, the study of *thoracic* horns has added a spectacular example underscoring the potential of how a deeply ancestral gene network, one best known for instructing the formation of insect wings, can fuel extensive innovation in insect development outside traditional wing-bearing segments. This now includes — besides beetle horns – the bilateral

projections of scarab pupae, *Tribolium* and *Tenebrio* gin traps, and very likely the helmets of membracid treehoppers as well as other bilateral and medial elaborations in the prothorax and abdomen of many insects. And just like *Antp* and *Ubx* play critical roles in the formation of fore- and hind wings, as does *Scr* in the formation of prothoracic beetle horns, Hox genes in general are likely to emerge as crucial players in the regulation and evolution of the segment-specific transcriptional landscapes that underpin the elaboration of a given wing serial homolog. Does this mean the prothoracic horns of beetles or helmets of treehoppers no longer count as morphological novelties? We posit that instead, discoveries such as these add to the growing call to – once and for all – abandon a definition of evolutionary novelty that necessitates the absence of homology and to instead focus on the genesis of novelty as a *gradual process of innovation* that allows descent with modification to seed the initiation of a novel trait, which once in existence can then diversify into its variant forms (Linz, Hu, and Moczek 2020). Viewed through the lens of such an *innovation gradient*, novelty emerges *through* homology, rather than somehow in its absence. Doing so then redirects our attention to begin investigating the taxonomic, anatomical, developmental-genetic, but also ecological conditions, and their interactions, that determine the circumstances in which ancestral homologies are enabled, or alternatively constrained, to fuel innovation.

The study of beetle *head* horns further confirms some of the same notions (Hu et al. 2020), but also necessitates additional nuances (Linz, Hu, and Moczek 2020). Rather than being instructed by the same core gene network underpinning the formation of wing serial homologs, the positioning and initiation of head horns appear to rely on the postembryonic re-deployment of embryonic head patterning mechanisms (Zattara et al. 2016a; Linz and Moczek 2020). If confirmed, this suggests that the evolutionary and developmental reach of the innovation gradient may be quite extraordinary: embryonic head patterning is among the most conserved of developmental genetic mechanisms across phyla, yet in *Onthophagus* beetles heterochronic changes in the interactions among select gene regulatory network components and their functions facilitated the origin of cephalic horns – all in rather evolutionarily recent times. The same work also suggested a possibly widespread yet easy to overlook mechanism that may facilitate the heterochronic redeployment of gene regulatory networks that execute key, conserved roles at earlier developmental stages: latent yet functionless expression. In both *Onthophagus* (Linz and Moczek 2020) and *Tribolium* (Zattara et al. 2016a), diverse postembryonic head patterning genes exhibit robust, regionally well-defined expression amenable to RNAi-mediated transcript depletion yet without resulting in any morphological effects in the dorsal head (though in many cases such effects are clearly discernable elsewhere in the body). Recall that one of these genes, *otd*, appears functionless in the dorsal head of adult *Tribolium*, but has acquired a critical new function in *Onthophagus* as a crucial regulator in the positioning of head horn. More generally, these and other results suggest that latent yet ancestrally functionless expression may act as a developmental scaffold for morphological innovation, allowing suites of genes with their (at least initially) region-specific expression and local interactors to become available for corresponding repurposing. Lastly, while *Onthophagus* head horns form in the dorsal head, and thus a body region generally assumed to be unaffected by Hox gene

function, a role of Hox genes cannot in fact be completely ruled out just yet. More generally, the broader significance of gene expression latency and the frequency of functional repurposing enabled by it and its contribution to the innovation gradient clearly deserve further scrutiny.

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