

THE ORIGIN AND DIVERSIFICATION OF COMPLEX TRAITS THROUGH MICRO- AND MACROEVOLUTION OF DEVELOPMENT: INSIGHTS FROM HORNED BEETLES

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Abstract

Understanding how development and ecology shape organismal evolution is a central goal of evolutionary developmental biology. This chapter highlights a class of traits and organisms that are emerging as new models in evo-devo and eco-devo research: beetle horns and horned beetles. Horned beetles are morphologically diverse, ecologically rich, and developmentally and genetically increasingly accessible. Recent studies have begun to take advantage of these attributes and are starting to link the microevolution of horned beetle development to the macroevolution of novel features, and to identify the genetic, developmental, and ecological mechanisms, and the interactions between them, that mediate organismal innovation and diversification in

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Current Topics in Developmental Biology, Volume 86
ISSN 0070-2153, DOI: 10.1016/S0070-2153(09)01006-0

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natural populations. Here, I review the most significant recent findings and their contributions to current frontiers in evolutionary developmental biology.

1. INTRODUCTION

Organismal form and function emerge during ontogeny through complex interactions between genotype, environmental conditions, and ontogenetic processes (Raff, 1996; West-Eberhard, 2003). These interactions are central themes in many biological and medical disciplines, and occupy a particularly prominent position in evolutionary biology: ultimately, evolutionary diversification of organismal form and function is possible only through changes in the nature of at least some of these interactions. This poses a particular challenge in the origin and diversification of novel, complex traits. Evolutionary novelties not only beg the question as to how they are made during ontogeny, but also how whatever it takes to make them was able to arise from whatever genetic and developmental tool box existed in the ancestor prior to their first origin. As outlined below, beetle horns and horned beetles offer an unusual opportunity to integrate genetic, developmental, physiological, and environmental mechanisms into a holistic understanding of how complex traits are generated, integrated, and modified during both development and evolution. In this chapter, I highlight and synthesize recent advances in our understanding of the genetic, developmental, and ecological origins of horns and horn diversity, as well as their consequences for diversification and radiation of horned beetles. Before doing so, however, I will briefly review what it is about beetles and their horns that makes them a promising window into the mechanics of innovation and diversification in nature.

2. UNIQUENESS AND DIVERSITY OF HORNS

Beetle horns combine several characteristics that make them outstanding models for exploring the origin, integration, and diversification of novel traits. First, beetle horns are massive, solid, three-dimensional outgrowths that often severely transform the shape of whoever bears them (Fig. 6.1; e.g., Mizunuma, 1999). Horns are often as long or longer than other appendages such as legs, can double the length of an individual, and can make up more than 30% of body mass. Not surprisingly, beetle horns often dominate the morphological and behavioral phenotype of their bearers. Second, beetle horns are *unique* structures lacking clear homology to existing traits in insects. They are not modified mouthparts or legs; instead, they exist *alongside* these structures in body regions in which insects normally do



Figure 6.1 Examples of horned beetles illustrating diversity and magnitude of horn expression in adult beetles. Clockwise from top: *Trypoxylus (Allomyrina) dichotoma*, *Onthophagus watanabei*, *Golofa claviger*, and *Phanaeus imperator*.

not produce any outgrowths (Moczek, 2005). Hence, horns can be looked at as an evolutionary innovation that occurred at some point during the history of beetles and which fueled one of the most impressive radiations of secondary sexual traits known in the animal kingdom. It is the resulting diversity of horn phenotypes that adds a third major rationale for horned beetles' utility as a model system for understanding the origins of organismal diversity (Arrow, 1951). Horn expression is restricted to relatively few beetle families such as the Tenebrionidae, Staphilinidae, Passalidae, Curculionidae, Chrysomelidae, and Scarabaeidae (reviewed in Moczek, 2005). However, within these families, and especially within the family

Scarabaeidae, horn expression is frequent and highly diverse. Moreover, much diversity can be found over remarkably short phylogenetic distances. For example, in the scarab genus *Onthophagus* species differ in the body regions that participate in horn growth (e.g., head or thorax), differ in horn number (single, paired, or combinations thereof), or differ in how horns scale with body size (e.g., isometric or sigmoidal; Balthasar, 1963). Amazingly, much of this diversity is also found within species where it is manifest in the expression of dramatic sexual dimorphisms as well as alternative male phenotypes (male dimorphism), suggesting possibly important links between the origins of diversity that exist within species to those existing between. This diversity in horn expression among conspecifics and congeners thus provides a remarkable opportunity to identify genetic and developmental mechanisms that generate variation in horn growth between individuals, as well as the ecological and behavioral causes that ultimately underlie this variation. To appreciate these causes, however, we must first learn more about the ecology of horned beetles and understand what, if anything, they use their horns for (Fig. 6.2).

3. FORM AND FUNCTION

Several hypotheses have been proposed to explain the adaptive significance of beetle horns (reviewed in Arrow, 1951). Horns have been thought to allow beetles to defend themselves against predators, indicate male quality to choosy females, or facilitate digging through soil. Alternatively, Arrow (1951) suggested that horns may actually have no function and may simply be the product of selection toward larger body size. However, little evidence exists in support of any of these hypotheses. In contrast, much evidence has now accumulated across a range of beetle families that suggests that horns are used as weapons in male–male combat over access to females (Cook, 1990; Eberhard, 1978; Emlen, 1997; Moczek and Emlen, 2000; Palmer, 1978; Siva-Jothy, 1987). Specifically, depending on horn size, shape, and fighting context, beetles use their horns to push, prod, lift, grab, stab or otherwise reduce their rivals' ability to access nearby females. For example, males of many species, including all members of the genus *Onthophagus* studied so far, fight in subterranean tunnels to gain or maintain access to breeding chambers and females. Here, fights take place within a confined space and horns are predominantly used as blocking and positioning devices. Fights then consist primarily of shoving contests, which can take a long time and appear energetically expensive injuries are rare to absent (Emlen, 1997; Moczek and Emlen, 2000; Palmer, 1978). In contrast, many species in the subfamily Dynastinae, such as the famous *Chalcosoma* species, fight arboreally. Here, horns are used to dislodge, lift, and throw



Figure 6.2 Diversity between and within *Onthophagus* species. (A) Six *Onthophagus* species illustrating the diversity of horn types that exist within the genus. (B) Sexual and male dimorphism in *Onthophagus nigriiventris*.

rivals off of branches. Fights can be brief but have the potential to inflict severe injury when males crack their exoskeleton upon hitting the ground (Beebe, 1944; Siva-Jothy, 1987). Both direct behavioral observations on several species (Emlen, 1997; Moczek and Emlen, 2000) and fitness estimates on at least one species (Hunt and Simmons, 2001) confirm that horn possession is indeed adaptive in these aggressive contests and improves a given male's chances of succeeding in fights.

Not all males within a species, however, express a full set of horns. In fact, horn dimorphisms are common in natural populations, resulting in the occurrence of two relatively discrete horned (also called major) and hornless (minor) morphs. Importantly, these alternative male morphs do *not* reflect allelic variants but instead are the product of environmental

differences—predominantly larval feeding conditions (Emlen, 1994; Moczek and Emlen, 1999). Larvae with access to optimal feeding conditions eclose to adult larger than a certain size threshold and thus express a full set of horns, whereas larvae limited to suboptimal conditions eclose at smaller adult sizes and remain largely hornless. This horn polyphenism is not restricted to morphological differences, but also results in discrete behavioral and physiological differences between morphs. For example, in contrast to the aggressive fighting behavior employed by horned males, small hornless males employ nonaggressive sneaking behaviors to access females (Moczek and Emlen, 2000). Similarly, hornless males produce disproportionately larger ejaculate volumes during copulation (Simmons *et al.*, 1999), and recent work also showed that the presence or absence of horns has profound consequences for individual thermoregulatory properties (Shepherd *et al.*, 2008).

In summary, the horns of beetles represent an evolutionary novelty of extraordinary diversity within and between species, and play a significant role in the behavioral ecology of individuals as well as populations. We are now in a good position to begin exploring the developmental and genetic basis of horns, an effort that began only a few years ago (Moczek and Nagy, 2005), but that has already yielded important insights into the origins of novel features.

4. THE ONTOGENY OF HORNS

Beetles are holometabolous insects, and as such the larval stage constitutes their main feeding stage. In general, the larvae of horned beetles use relatively low-nutrition food sources such as dung (e.g., *Onthophagus*), decaying plant matter (e.g., *Chalcosoma*, *Trypoxylus*), or carrion (e.g., *Coprophanaeus*), and depending on the final adult size, larval development may take anywhere from weeks (*Onthophagus*) to several years (*Chalcosoma*). With respect to horn development, however, little happens during this period. Instead, most if not all horn patterning and growth takes place very late in larval development when the animal is nearing the transition to the pupal stage. Here, two brief and temporally dissociated stages are primarily responsible for generating and differentiating horn primordia during development. The transient *prepupal* stage at the very end of larval development marks the first of these two. At this point, all larval epidermis detaches from the larval cuticle—a phenomenon known as apolysis—and selected regions in the head and/or thorax undergo dramatic cell proliferation to generate the pupal precursors of adult horns (Moczek and Nagy, 2005). The *pupal* stage then marks the onset of the second developmental phase important for adult horn expression. During this stage, the pupal epidermis apolyses once more, but instead of the rapid growth marking

earlier stages, apolysis is followed by sculpting and remodeling of the pupal epidermis into the final adult shape. Remodeling can be dramatic and is capable of removing large amounts of pupal horn tissue over a period of just a few days. In many species, pupal remodeling allows fully horned pupae to molt into entirely hornless adults (Moczek, 2006b). Degree of horn expression among adult beetles is thus the consequence of both prepupal growth late in larval development and the pupal remodeling phase just prior to the final, adult molt. Importantly, even congeneric species can differ widely in the degree to which they rely on one or the other mechanisms in generating intra- and interspecific diversity.

More generally, however, beetle horns originate and differentiate in a manner surprisingly similar to the primordia of adult legs, mouthparts, wings, or antennae of most insect orders (Svácha, 1992). Like horns, traditional appendages such as legs and mouthparts are epidermal outgrowths that form during late larval development and are remodeled during the pupal stage. The only dramatic deviation from this pattern occurs in all appendages produced by higher flies as well as in the wings of Hymenoptera, Lepidoptera, and some Coleoptera (Svácha, 1992). In these cases, appendages develop from imaginal disks, which represent a highly derived mode of appendage formation absent in the majority of insect orders (Kojima, 2004). Imaginal disks are epidermal invaginations specified during embryonic development which grow throughout larval development. Moreover, many important patterning steps take place while the disk is essentially a two-dimensional sheet of tissue, and all disk growth occurs while the disk is *invaginated* into the body interior (Fig. 6.3A). Beetle horns differ in that they (a) appear not to be specified during embryonic development, (b) grow from the start as three-dimensional epidermal outbuddings, (c) have their growth confined to the relatively brief prepupal stage (~48 h), and (d) as they grow, evaginate into the space between epidermis and larval cuticle (Moczek, 2006a; Fig. 6.3B). Consequently, the *Drosophila* model of limb development has likely limited applicability for beetle horns. Instead, given their growth as epidermal outbuddings, beetle horns develop more like the appendages of most other insect orders (Svácha, 1992). Unfortunately, most of our understanding of insect appendage formation comes from studies of imaginal disk development in *Drosophila* (Kojima, 2004). Consequently, even though faced with serious limitations when applied to beetle horns, the *Drosophila* model of limb development represents our best starting point to begin exploring the regulation of horn growth and differentiation (Fig. 6.4).

5. THE REGULATION OF PREPUPAL HORN GROWTH

As introduced above, one way to think of beetle horns is as simplified appendages. Unlike traditional appendages, beetle horns lack muscles, nerves, or joints, but like traditional appendages, beetle horns are three-dimensional

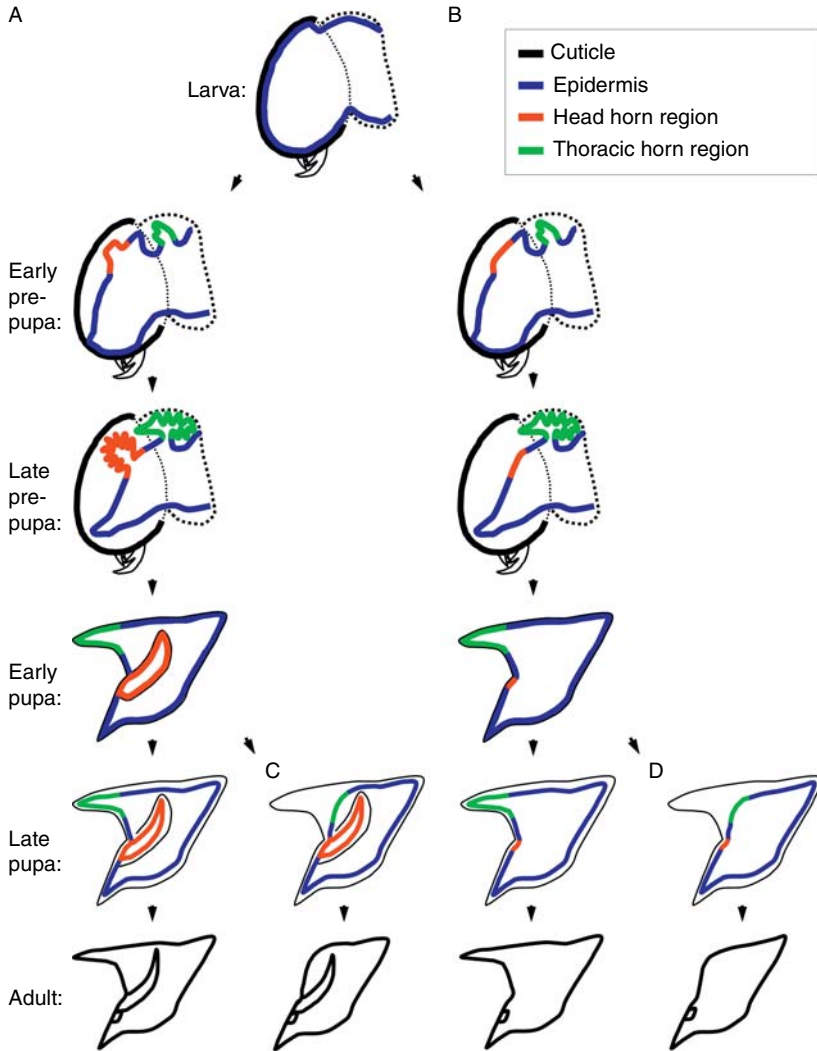


Figure 6.3 Development of horns and horn dimorphisms in *Onthophagus* beetles. (A) Apolysis is followed by rapid cell proliferation of selected epidermal tissue regions (shown here for a head horn and thoracic horn). Horn primordia expand during the pupal molt and become externally visible. During the pupal stage epidermal cells apolyse once more, followed by remodeling of the pupal epidermis into the final adult shape. The pupa then undergoes one last molt to the final adult stage. (B) Development of horn dimorphisms through differential proliferation of prepupal horn tissue (illustrated here for head horns). During the prepupal stage presumptive horn tissue proliferates little, resulting in the absence of external horns in pupae and resulting adults. (C, D) Development of horn dimorphisms through differential remodeling of pupal horn tissue (illustrated here for thoracic horns). Pupal horn epidermis is resorbed prior to the secretion of the adult cuticle. This mechanism generates sexual dimorphisms for thoracic horns in many species, and can occur in the presence or absence of (differential) head horn development (modified after Moczek, 2005).

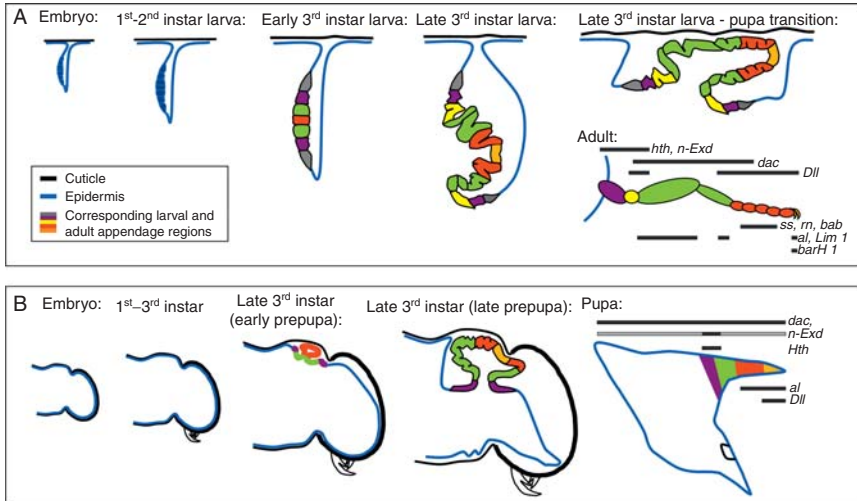


Figure 6.4 Differences and similarities in the development of the (A) *Drosophila* leg and (B) thoracic horns in beetles (see text for details). Colors indicate tissue types and regional relationships between immature and mature appendage. Also indicated is the approximate relationship between expression domains of common p/d patterning genes during development and the corresponding adult appendage region (modified after Moczek, 2006a,b).

outgrowths of epidermal origin with clearly defined proximodistal, medio-lateral, and anterior–posterior polarities. This raises the possibility that the regulation of beetle horns may rely at least in part on the same genetic and developmental mechanisms that regulate the expression of more traditional appendages. Recent data, focusing on the establishment of the proximodistal (p/d) axis, strongly support this hypothesis.

In *Drosophila*, establishment of the proximodistal axis begins with the concentration-dependent and combined action of two diffusible morphogens: *wingless* (*wg*) and *decapentaplegic* (*Dpp*). These subdivide imaginal disks into roughly concentric, nested domains of expression of several transcription factors including *Distal-less* (*Dll*), *dachshund* (*dac*), and *homothorax* (*hth*). The center of the leg disk, characterized by *Dll* expression, eventually gives rise to the distal region of the adult appendage, while progressively more peripheral disk regions, characterized by *dac* and *hth* expression, form progressively more proximal appendage regions once the imaginal disk telescopes outwards to form the adult appendage (reviewed in Kojima, 2004). As emphasized above, in most other insects, adult appendages develop not from imaginal disks but via the outbudding of selected epidermal regions during larval development (e.g., Fristrom and Fristrom, 1993; Nagy and Williams, 2001). Despite these fundamental differences in the

morphogenesis of appendages, there remain many similarities in the underlying patterning mechanisms. For example, *Dll* expression in the distal region and *hth* expression in the proximal region occurs during the development of appendages in a wide range of insects and noninsect arthropods (Abzhanov and Kaufman, 2000; Inoue *et al.*, 2002; Jockusch *et al.*, 2000; Mittmann and Scholtz, 2001; Prpic and Tautz, 2003; Suzuki and Palopoli, 2001), and *Dll* activity is functionally required for distal leg formation in beetles and spiders (Beermann *et al.*, 2001; Schoppmeier and Damen, 2001). *Dll*, *dac*, and *hth* therefore represented legitimate candidate genes for the regulation of p/d axis formation and growth during beetle horn development (Fig. 6.5).

Expression studies lend first support to an involvement of at least two, and possibly all three of these transcription factors during horn development (Moczek and Nagy, 2005; Moczek *et al.*, 2006). In several *Onthophagus* species, *Dll* expression was found in the regions of prepupal horn primordia that later would form the part of the adult horn, while *hth* expression was confined to incipient proximal horn regions. In contrast, *dachshund*, a transcription factor normally involved in patterning medial appendage identity clearly violated the *Drosophila* model and was expressed well outside its predicted medial domain. These results suggested that partial redeployment of p/d patterning genes may have played a role in the origin of beetle horns, however, in the absence of functional assays any extrapolation from gene expression to gene function had to remain tentative at best. Recently, larval RNA interference (RNAi)-mediated gene function analyses have been used to further examine possible roles of *dac*, *hth*, and *Dll* in beetle horn development, with many interesting results (Moczek and Rose, unpublished data).

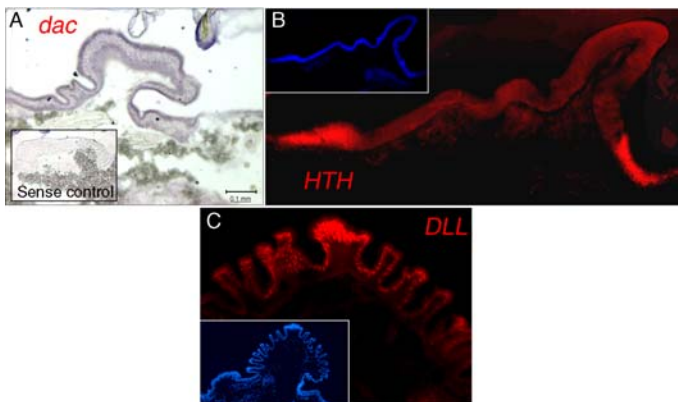


Figure 6.5 Examples of p/d genes expressed during horn development. (A) *Dachshund* *in situ* hybridization of the transient thoracic horn primordium in *O. taurus*. (B) Anti-HTH immunostaining of the persisting thoracic horn primordium of *O. binodis*. (C) Anti-DLL immunostaining of one of two head horn primordia in *O. taurus*.

First, irrespective of any involvement in horn development, larval RNAi-mediated transcript depletion of all three patterning genes generated phenotypic effects very similar to those documented by previous studies. For instance, *Dll* and *dac*RNAi resulted in loss or fusion of distal and medial regions, respectively, in the leg and antenna (Angelini and Kaufman, 2004; Kojima, 2004; Prpic *et al.*, 2001). Similarly, *hth*RNAi accelerated eye differentiation (Bessa *et al.*, 2002) and induced ectopic wing tissue on the first thoracic segment (Ryoo *et al.*, 1999; Yao *et al.*, 1999). These results documented for the first time both the feasibility and power of RNAi-mediated gene knockdown in horned beetles. In addition, this study yielded the first functional insights into the regulation of horn development. Specifically, the study showed that despite being widely expressed throughout prepupal horn primordia in *Onthophagus* (Moczek *et al.*, 2006) *dac* does not appear to play any obvious role in the regulation of size, shape, or identity of horns. Instead, *Otdac*RNAi individuals expressed thoracic and head horns of precisely the same size and overall shape as control animals despite severe *dac* knockdown phenotypes elsewhere in their body. In contrast, *hth* transcript depletion had a dramatic effect on horn expression, but only in one horn type: thoracic horns. *hth* transcript depletion resulted in drastically shortened thoracic horns over the entire range of body sizes, but had no effect on head horn expression. Instead, *Othth*RNAi individuals expressed head horns indistinguishable from control individuals despite severe effects on other head appendages. The results of *Dll*RNAi complicated things even further. Unlike *hth*, *Dll* transcript depletion affected the expression of both head and thoracic horns, but not in the same individuals or even species. In *Onthophagus taurus*, head horn expression was only affected in large males otherwise fated to express a full set of head horns, whereas horn expression in small- and medium-sized males was unaffected, as was the expression of pupal thoracic horns in both males and females regardless of body size. In the congener *O. binodis*, however, *Dll*RNAi affected thoracic horn expression and did so in both males and females, though the effect was strongest in large individuals. Combined, these results suggest that *Onthophagus Dll* and *hth*, but not *dac*, alter horn expression in a sex-, body region-, and body size-specific manner, and that even closely related species can diverge rather substantially in aspects of this regulation (Fig. 6.6).

These results are the first to suggest that horn development evolved via differential co-option of at least some p/d patterning genes normally involved in traditional appendage formation. On one side, these results are not surprising and confirm a general phenomenon in the evolution of novel traits: new morphologies do not require new genes or developmental pathways and instead may arise by recruiting existing developmental mechanisms into new contexts. On the other, these results also highlighted an unexpected degree of evolutionary lability, ranging from the absence of

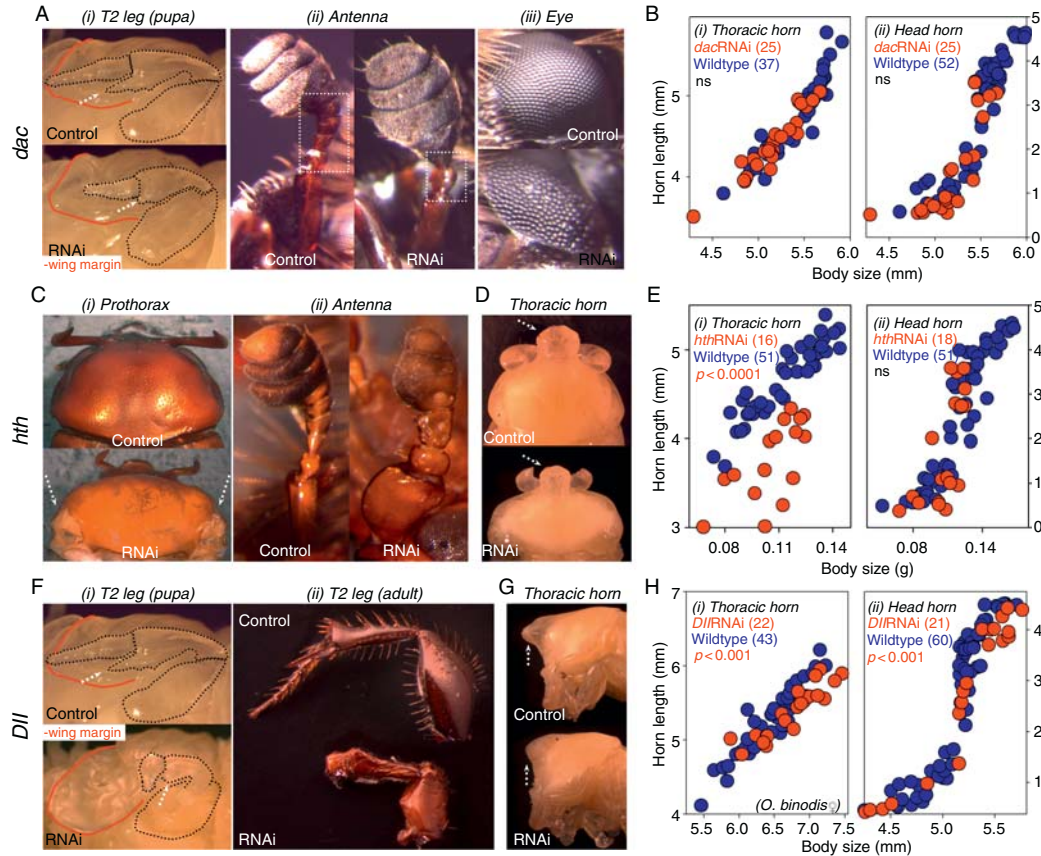


Figure 6.6 Larval RNAi-mediated transcript depletion of (A, B) *dachshund*, (C–E) *homothorax*, and (F–H) *Distal-less*. Images illustrate typical phenotypes observed in each experiment compared to wild-type phenotypes. Graphs depict scaling relationships between pupal body size and horn length for thoracic horns (i) and head horns (ii). Wild-type is shown in blue and RNAi-treated individuals are shown in red. All data are from male *O. taurus* except H(i) which were collected from female *O. binodis*. Sample sizes are given in parentheses (modified after [Moczek and Rose, unpublished data](#)).

patterning function (*dac*) to patterning function in selected horn types only (*hth*, *Dll*) to function in one size class, sex, or species but not another (*Dll*). Most specifically, this suggests that different horn types, and even the same horn type in different species, may be regulated at least in part by different pathways. Different horn types may therefore have experienced distinct, and possibly independent, evolutionary histories.

It is important to realize that this is clearly just the beginning of a more detailed analysis of the developmental regulation and diversification of beetle horns. The recent development of *Onthophagus* EST libraries now provides access to members of many regulatory pathways known to be involved in insect development, ranging from genes involved in axis specification, patterning, and morphogenesis, to genes involved in many prominent signaling pathways, to genes involved in endocrine regulation of development. Furthermore, *Onthophagus* microarrays developed from these libraries have added a critical tool for rapid comparative transcriptional profiling across species, sexes, morphs, and even tissue regions within individuals. Clearly, much work lies ahead before we will have achieved a solid understanding of beetle horn development and its similarities and differences to other developmental processes. However, the most critical resources are now in place that promise that this goal will be attained within the near future. It is due to these same resources that we are already beginning to gain a much better insight into the regulation of the second developmental stage crucial for adult horn expression: the pupal remodeling stage.

6. THE REGULATION OF PUPAL REMODELING

As introduced above, the pupal stage marks the second developmental period critical to defining the final size and shape of adult horns. During this stage, animals undergo the same basic developmental steps as during the previous molts such as apolysis of the epidermis, secretion of a new cuticle, and eclosion to the next developmental stage. However, unlike in previous molts, there is no proliferation stage, and horns, just like other body parts, do not grow significantly during the pupal stage. Secondly, in at least one horn type, those extending from the thorax, there is frequent differential loss, or resorption, of presumptive horn tissue. In such cases, fully horned pupae molt into thorax horn-less adults lacking any signs of the previous existence of a thoracic horn primordium. Of 19 *Onthophagus* species studied thus far, four species utilized differential, sex-specific resorption of thoracic horn tissue to generate sexual dimorphism. The remaining 15 species use the same process to remove thoracic horn primordia in *both* sexes. In at least one of those, *O. taurus*, pupal thoracic horn resorption *eliminates* a

pronounced sexual dimorphism in thoracic horns evident in pupae, but not in the resulting adults (Moczek *et al.*, 2006). Recent work now strongly implicates programmed cell death (PCD) in the destruction and removal of horn primordial tissue (Fig. 6.7).

PCD involves the coordinated destruction of cytoplasmic contents including organelles and their membranes as well as nuclear DNA degradation (Potten and Wilson, 2004). As such, PCD relies on a complex cascade of developmental and cellular processes. Despite this apparent complexity, PCD is an ancient physiological process employed by all metazoan organisms to dispose of cells during development. A recent study showed that primordial epidermis of horns programmed to be resorbed undergoes premature PCD during the first 24 h of the pupal stage (Moczek, 2006b). Relying on two different biochemical assays, the same study then showed that PCD is considerably more frequent among horn primordial cells of transient horns compared to individuals whose pupal horns are being converted into an adult structure, supporting the hypothesis that PCD is the most likely mechanisms by which horn resorption and remodeling are achieved. At the same time, comparisons across species suggested that the exact position and timing of PCD-mediated horn remodeling can differ remarkably from one species to the next. Combined, the regulation of pupal remodeling reveals many of the same features highlighted above for the regulation of horn growth. On one side, pupal remodeling and resorption of horns appears to rely on an ancient developmental mechanism, PCD, which has been recruited into a new developmental context. On the other, results suggest the existence of considerable variation within and between species regarding when, where, and how much remodeling of horns occurs. By extension, this variation suggests the existence of modifier mechanisms that regulate species-, sex-, and body region-specific resorption of horns. The identity and nature of these modifier mechanisms are currently being investigated, and many interesting preliminary data have already been collected (Fig. 6.8).

For instance, previous work on *Drosophila* has shown that the Hox genes *Deformed* (*Dfd*) and *Abdominal-B* (*Abd-B*) regulate segment boundaries through the regional activation of PCD (Lohmann *et al.*, 2002), suggesting regional Hox genes as possible gene candidates for the regulation of PCD-mediated resorption of pupal horns. Indeed, preliminary data on *Onthophagus* now suggest that the Hox gene *Sex combs reduced* (*Scr*), traditionally responsible for patterning the first thoracic segment in insects, has acquired the function to regulate PCD-mediated pupal horn remodeling, and that it exerts this function in a sex- and species-dependent manner (B. Wasik, D. Rose, and A. P. Moczek, unpublished data). Similarly, research on a variety of insects has shown PCD to be regulated by endocrine factors, in particular ecdysteroids and juvenile hormone (JH; e.g., Lobbia *et al.*, 2007; Oliver *et al.*, 2007). In *Onthophagus*, at least JH appears to play an important

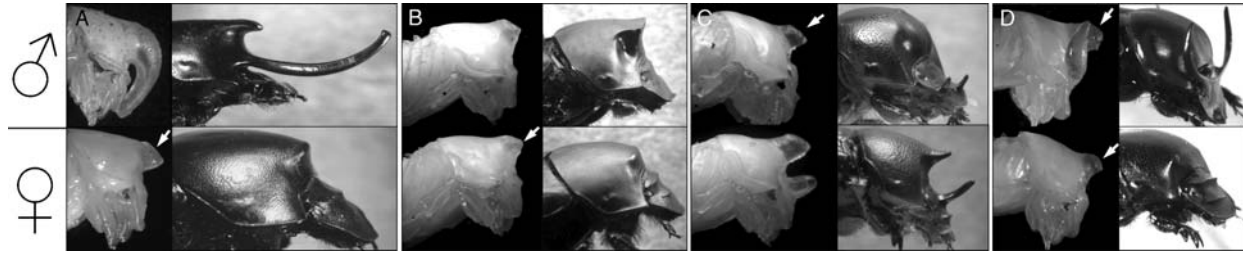


Figure 6.7 Pupal horn remodeling is common in the genus *Onthophagus* yet variable among species. Males (top) and females (bottom) of four *Onthophagus* species. Pupae are shown left and corresponding adults on the right. (A) *O. nigriventris*, (B) *O. binodis*, (C) *O. sagittarius*, and (D) *O. taurus*. Arrows highlight cases of pupal horn resorption.

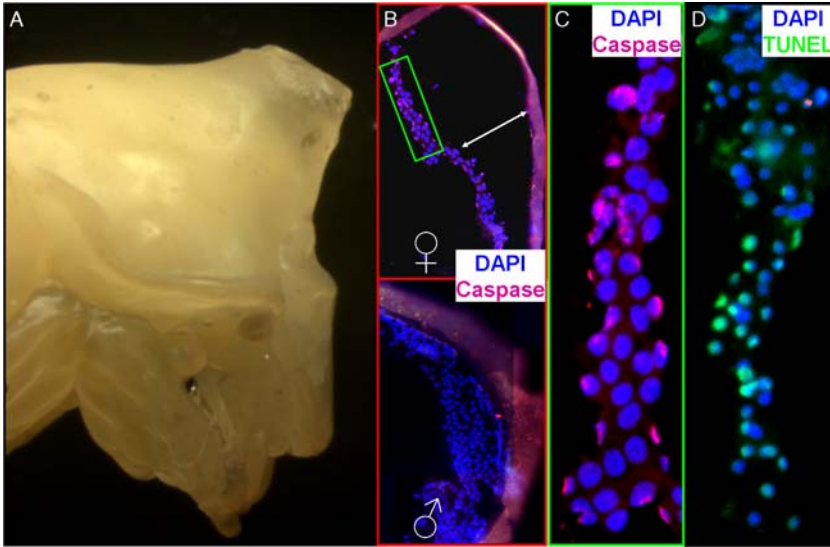


Figure 6.8 Programmed cell death appears to mediate sex-specific pupal remodeling in *O. binodis*. (A) Pupa indicating distal thoracic horn. (B) Anti-DRICE (activated caspase-3) staining in thoracic horn epidermis on pupal day 1 in (top) and (bottom). (C) Detail of Bè. (D) Corresponding region stained with TUNEL assay to detect PCD-specific DNA fragmentation.

role in the regulation of horn expression, and both sexes and species differ in degree and nature of changes in horn expression that are induced by the same JH manipulation (Emlen and Nijhout, 1999; Shelby *et al.*, 2007). In summary, while existing data clearly provide only a very superficial understanding of the developmental regulation and diversification of pupal remodeling of beetle horns, promising avenues for future research exist that should soon make this an exciting area of study.

7. THE REGULATION OF PLASTICITY

The horns of male beetles are as much famous for their extravagance and splendor as they are for the incredible variation in horn expression that exists between males of the same species. In fact, diversity between conspecific males often parallels differences between species, especially in cases in which discrete large, horned (major) and small, hornless (minor) morphs co-occur in the same population. In such instances, the existence of alternative morphs has occasionally resulted in them being described as different species (Paulian, 1935). As explained earlier, differences in body size and

horn expression between conspecific males, including the expression of discrete morphs, occur in response to differences in environmental conditions, especially larval feeding conditions (Emlen, 1994; Moczek and Emlen, 1999). Larvae with access to optimal feeding conditions eclose to larger body sizes and express larger, and often disproportionately larger, horns, whereas larvae with access to poorer conditions eclose to smaller adult sizes and express no or greatly reduced horns. This *plasticity* in body size and horn expression gives rise to particular allometric scaling relationships between body size and horn length. Such allometries can differ dramatically between species and sexes, ranging from isometric allometries (in species in which large males are proportionally enlarged versions of smaller males) to positive allometry (in species in which large male have disproportionately enlarged horns compared to small males) to sigmoidal allometries (in species in which alternative morphs are separated by a threshold size). Importantly, closely related species and even populations of the same species can diverge in aspects of these scaling relationships such as the allometric slope or body size threshold separating alternative morphs (Moczek and Nijhout, 2003; Moczek *et al.*, 2002). This suggests that even though the individual differences in body size and horn length are brought about by environmental differences, heritable variation exists between genotypes causing them to respond differently to the same nutritional variation. Selection or drift can then act on this variation and shape species-specific allometries in nature. For instance, a particularly illuminating case of allometric divergence has been documented in *O. taurus*, in which three exotic populations established less than 50 years ago from the native range of the species have diverged heritably in body size thresholds to a degree normally only observed between species (Moczek and Nijhout, 2003). Ecological studies in the field suggest that these divergences were driven by differences in the intensity of intra- and interspecific competition for breeding opportunity, and thus changes in the social context within which horned (fighting) and hornless (sneaking) male morphs function (Moczek, 2003). More generally, these and other findings highlight that besides the origin and diversification of horns *per se*, the evolution and diversification of *plasticity* in horn expression has contributed massively to extant patterns of morphological diversity. This implies the existence of independent regulatory mechanisms which, rather than controlling the expression of horns, regulate the degree and nature of *plasticity* in horn expression in response to environmental gradients. *Onthophagus* beetles again provide outstanding opportunities to explore the developmental and genetic regulation of *plasticity* given the enormous variation in *plasticity* of horn expression that exists between species, ranging from absence of environmental sensitivity to complete determination by nutritional conditions. In some cases, both extremes of sensitivity can even be found in different horn types expressed by *the same individual*, such as the nutrition-insensitive

pupal thoracic horns of male *O. taurus* and the highly nutrition-sensitive head horns in the same individuals. Identifying the nature and mechanics of developmental pathways underlying plasticity in horn expression, and evolutionary changes in horn expression, are therefore major foci of current research, and several important insights have already been gained.

For instance, comparing the two most divergent *O. taurus* populations mentioned above, Moczek and Nijhout (2002) found that allometric divergences correlated with evolved differences in degree and timing of sensitivity to JH. In both populations, artificial applications of a JH analog induced horns in male larvae otherwise fated to eclose into hornless adults. However, populations in which males already expressed horns at relatively small body sizes were more sensitive to JH manipulations, and were sensitive *earlier* in development compared to populations that confined horn expression to only but the largest males. These findings supported the hypothesis that a JH titer-mediated threshold response underlies the expression of alternative male morphs. Moreover, it suggested that aspects of this threshold response, such as degree and timing of sensitivity to JH, are capable of undergoing remarkably rapid evolution in natural populations. A recent study by Shelby *et al.* (2007) extended this perspective to sexual dimorphisms and interspecific differences in horn size and shape.

These and other observations therefore suggest that endocrine factors such as JH mediate between nutritional variation experienced by larvae and morphological, behavioral, and physiological variation that exists among the resulting adults. The mechanisms by which endocrine factors adjust development to environmental conditions are presently not understood, but many critical resources exist that will allow researchers to make headway in this direction over the next few years. For instance, the *Onthophagus* cDNA libraries and microarrays introduced earlier contain *Onthophagus* orthologs of many genes involved in a plethora of developmental processes likely to be crucial for horn formation as well as many genes likely involved in endocrine regulation via ecdysteroid-, JH-, and Insulin-signaling. Studies are now under way to use these and other resources to identify genes, pathways, and gene networks whose expression change in response to nutritional changes, to characterize the level of conservation of this induction across body regions, morphs, sexes, and species, and ultimately to identify the functions of the most promising gene candidates.

8. THE ORIGINS OF NOVELTY AND DIVERSITY

The preceding sections highlighted several areas in which we are beginning to get a better understanding of the regulation of developmental processes relevant to growth and differentiation of horns, such as the

function of p/d axis patterning genes during prepupal growth, the activation of PCD during pupal remodeling, or the endocrine underpinning of plasticity in horn expression. Each of these cases illustrates a by-now-familiar pattern in the evolution of development, including the evolution of novel features: novel traits do not require new genes or developmental pathways to come into being, but instead may arise from co-option of pre-existing developmental machinery into new contexts. P/d patterning genes and PCD still carry their ancestral function of instructing axis polarity or removing superfluous cells, but what is new is the location and timing of their action. Further research into the regulation of beetle horn development will undoubtedly add additional examples. In addition, we are also beginning to see examples of possibly truly novel functions, acquired by old regulators during the evolution of beetle horns. For example, if current research further confirms that the Hox gene *Scr* regulates PCD during the pupal remodeling phase of development, this may well emerge as a regulatory function of *Scr* that is unique to horned beetles and which has no parallels to its ancestral functions during insect development. If correct, this would suggest that the evolution of beetle horns involved the recruitment of conserved developmental mechanisms into new contexts enriched by novel regulatory interactions acquired by pre-existing regulatory genes. Lastly, we do not yet know of any genes or pathways whose expression and functions are entirely unique to beetle horns, but we should not lose sight of this possibility. Current *Onthophagus* arrays contain several hundred ESTs with large open reading frames yet lacking obvious orthology to existing databases, and it is conceivable that some of those may represent horn-specific genes and regulators that evolved solely in the context of *Onthophagus* horn development. In addition to identifying conserved or putatively novel regulatory properties of interesting genes and pathways, the studies on beetle horn development summarized above have also unearthed tremendous *variation* in these properties between morphs, sexes, populations, and species. Combined, these findings have three major implications. First, they contradict the notion that highly upstream regulators, such as p/d patterning genes or Hox genes, should be evolutionarily entrenched given their importance in the regulation of basic aspects of animal architecture and thus resistant to the acquisition of novel functions (Davidson and Erwin, 2006). Instead, they illustrate that regulatory genes whose functions are otherwise highly conserved nevertheless retain the capacity to acquire additional functions. Second, results to date suggest that little phylogenetic distance is necessary for the evolution of sex- and species-specific differences in these functions. If confirmed, this would argue that even master-regulator genes and their interactions can diversify on the level of populations and species with unexpected ease. Third, many of the developmental differences seen between species, such as the presence or absence of horns or horn expression in different body regions, have striking parallels in sexual

dimorphisms or male dimorphisms. This raises the possibility that the developmental capacity to generate macroevolutionary differences may originate well within species, between sexes, and—fueled by developmental plasticity—across alternative morphs.

Understanding the developmental, behavioral, and ecological basis of horns and horn diversity, however incomplete, now puts us in a position to address the questions posed at the beginning of this chapter. What are the genetic, developmental, and ecological mechanisms, and the interactions between them that brought about the first transition from a hornless ancestor to a horned descendant, and that since have shaped the subsequent diversification of beetle horns? Recent work has begun to provide some surprising answers to both of these questions, and I will end this chapter by highlighting where we have made the most progress. I will begin, appropriately, with the origin of horns. Specifically, I will focus on one particular horn type, those protruding from the thorax, where recent work has made the greatest headway toward understanding the possible origin of these structures.

9. THORACIC HORNS AS AN EXAPTATION

As introduced above, PCD-mediated resorption of pupal thoracic horn primordia appears common, if not ubiquitous, among *Onthophagus* species, raising the question as to the adaptive significance, if any, of transient horn expression. Experimental approaches have now revealed that pupal horns, irrespective of whether they give rise to a corresponding adult structure or not, actually play a crucial role during the larval-to-pupal molt and the shedding of the larval head capsule (Moczek *et al.*, 2006). Unlike in larval-larval and pupal-adult molts, larvae that molt into pupae have little muscle tissue left that could aid in the shedding of the larval cuticle, as most larval muscles have already undergone histolysis. Instead, the animal uses peristaltic contractions to pump hemolymph to inflate selected body regions and to force old cuticle to rupture. This is sufficient to remove the highly membranous thoracic and abdominal cuticle of larval scarab beetles. However, the larval head capsule may pose additional challenges as it is composed of extremely thick cuticle used between molts to anchor powerful jaw muscles. Such muscles, and the corresponding head capsule, may be particularly strong in fiber-feeding scarab larvae such as *Onthophagus*, and this is where the thoracic horn primordia of *Onthophagus* beetles unexpectedly enter the stage. Carefully staged sections showed that during *Onthophagus*' prepupal stage, thoracic horn primordia insert themselves into the space vacated between the larval head capsule and corresponding epidermis and subsequently fill with hemolymph and expand. Eventually,

this expansion forces the larval head capsule to fracture along prepatterned suture lines. As a consequence, as the larval head molts into a pupal head, the first pupal structure visible from the outside is not a part of the head, but instead the thoracic horn primordium as it breaks through the head capsule. Experimental elimination of thoracic horn primordia prior to the prepupal stage resulted in pupae that (a) lack a thoracic horn and (b) failed to shed their larval head capsule (Moczek *et al.*, 2006). Replicating this approach in and outside the genus *Onthophagus* showed that this putative dual function of thoracic horn primordia appears unique to onthophagine beetles. Further phylogenetic analyses suggested that the pupal molting function of horns preceded the horns-as-a-weapon function of the adult counterparts, and that ancestrally, pupal horns were always resorbed prior to the adult molt (Moczek *et al.*, 2006). If correct, this would explain why so many *Onthophagus* species grow thoracic horns even though those outgrowths are not used to form a functional structure in the adult.

These results also raise the possibility that the origin of *adult* horns could have been the result of a simple failure to remove otherwise pupal-specific projections through PCD. A survey of the available literature suggests that such events actually occur in natural populations frequently enough to be detected by entomologists (e.g., Ballerio, 1999; Paulian, 1945; Ziani, 1994). Even though such an outgrowth would initially have been rather small, behavioral studies have shown that if used in the context of a fight, even very small increases in horn length bring about significant increases in fighting success and fitness (Emlen, 1997; Moczek and Emlen, 2000). Behavioral studies have also shown that aggressive fighting behavior is widespread among beetles and occurs well outside horned taxa. Possession of adult horns is therefore not a prerequisite for fighting, instead male beetles most likely fought each other well before the first adult horn ever surfaced, creating a selective environment in which the first pupal horn that failed to be removed before the adult molt could have provided an immediate fitness advantage. Thoracic beetle horns may thus be a good example of a novelty that arose as an exaptation from traits originally selected for providing a completely different function during a completely different stage of development. It is equally important to realize, however, that none of these arguments appear to hold for other horn types such as head horns. Head horns, at least in *Onthophagus*, only undergo mild remodeling if any, and morphological differences among adults are already largely established in the preceding pupal stage (Moczek, 2007). These basic differences underscore the likely evolutionary and developmental independence that characterizes different types of horns, and most likely different lineages of horned beetles. More generally, the possible origin of adult thoracic horns from ancestral molting devices provides a vivid example of the crooked routes that developmental evolution is capable of taking as it generates what we in the end perceive as an evolutionary novelty. The same complexity in

the interactions between development, morphology, and ecology emerges when we examine the diversification of already existing horns, as the next example hopes to illustrate.

10. DEVELOPMENTAL TRADEOFFS AND THE DIVERSIFICATION OF HORNS AND HORNED BEETLES

Holometabolous insects such as beetles provide an exceptional opportunity to study a phenomenon believed to have shaped phenotypic diversity well beyond the insects and that is likely important for all metazoan organisms: resource allocation tradeoffs during development. Resource allocation tradeoffs arise during development when two or more structures compete for a shared and limited resource to sustain their growth. As such, resource allocation tradeoffs not only have the potential to alter ontogenetic outcomes, as developmental enlargements of one structure may only be feasible at the expense of another, but also evolutionary trajectories, as development may only be able to accommodate evolutionary enlargements of one structure through compensatory reduction of another. Resource allocation tradeoffs are likely ubiquitous during metazoan development, but are possibly particularly important in the development of holometabolous insects. Here, all growth of adult structures is confined to a time period during which larvae no longer take in nutrients and so represents essentially a closed system with a finite pool of resources to fuel all of metamorphosis. While the exact nature of resource allocation tradeoffs remains obscure, growing evidence exists that they have real potential to bias developmental outcomes and long-term evolutionary trajectories (Nijhout and Emlen, 1998). Recent work on horned beetles has begun to implicate resource allocation tradeoffs in the diversification of horns and other body parts, with intriguing implications for the diversification of horned beetle species (Emlen, 2001; Kawano, 2002; Moczek and Nijhout, 2004; Parzer and Moczek, 2008; Simmons and Emlen, 2006; Simmons *et al.*, 2007).

In 2002, Kazuo Kawano showed that two species of giant rhinoceros beetles (genus *Chalcosoma*) had diverged in both relative horn sizes and copulatory organ sizes, and that this divergence was more pronounced between sympatric (overlapping) than allopatric (separated) populations. His findings were perfectly consistent with reproductive character displacement reinforced in sympatry but not allopatry. What was intriguing, however, was the observation that the species which had evolved relatively longer horns had also evolved relatively shorter copulatory organs, and vice versa. In other words, male horn sizes and copulatory organ sizes had coevolved *antagonistically*. Subsequent experimental work on *O. taurus*

(Moczek and Nijhout, 2004) suggested that this antagonistic coevolution may not have been a coincidence. In this study, surgical ablation of the genital precursor tissue during development resulted in males with disproportionately longer horns. The magnitude of the effect depended on timing of ablation, which contradicted an earlier study that emphasized physical proximity as the main determinant of tradeoff intensity (Emlen, 2001). Rather than growing close to each other, it seemed that growing at the same time was more important in determining whether tradeoffs would occur or not. These arguments aside, the available data suggested that there may be a connection between how horns and copulatory organs developed, and therefore how they evolved. This was particularly intriguing because changes in male copulatory organs are thought to play a major role in the evolution of reproductive isolation, and thus, speciation (Eberhard, 1985). In fact, copulatory organ morphology is often the only way to distinguish cryptic and recent species, suggesting that whatever mechanism is able to influence copulatory organ expression in a population may have immediate repercussions for that population's ability to interbreed with others.

The strongest evidence to date that suggests exactly that kind of interaction between horn evolution and copulatory organ evolution now comes from a very recent study examining both within- and between-species covariation in horn investment versus copulatory organ investment (Parzer and Moczek, 2008). Specifically, this study focused first on the rapidly diverging exotic *O. taurus* populations introduced earlier. Recall that these populations were introduced from their native Mediterranean range to the Eastern US as well as to Eastern and Western Australia, and that these introductions occurred less than 50 years ago. Also, recall that all of these populations have evolved significant differences in male horn investment due to diversifying selection acting on the horn-length switch point, with Western Australian males growing the relatively shortest horns whereas Eastern US males grow the relatively longest, with the other two populations intermediate (Moczek, 2003; Moczek and Nijhout, 2003). Add to this the realization that there is no sympatry between any of these populations, and you have the perfect test situation to answer whether evolutionary changes in horn investment may cause correlated changes in copulatory organ size independent of possible reproductive character displacement in sympatry. And the answer is: they do! Among the four populations examined, there was a perfect negative correlation between relative investment into horns and relative investment into copulatory organ size. As a second step, the study applied the same approach to nine different *Onthophagus* species, and the same highly significant negative correlation between relative investment into horns and copulatory organ size emerged. Intriguingly, the greatest differences observed between *O. taurus* populations were similar in nature and magnitude to some of the differences detected between *Onthophagus* species. These results had three major

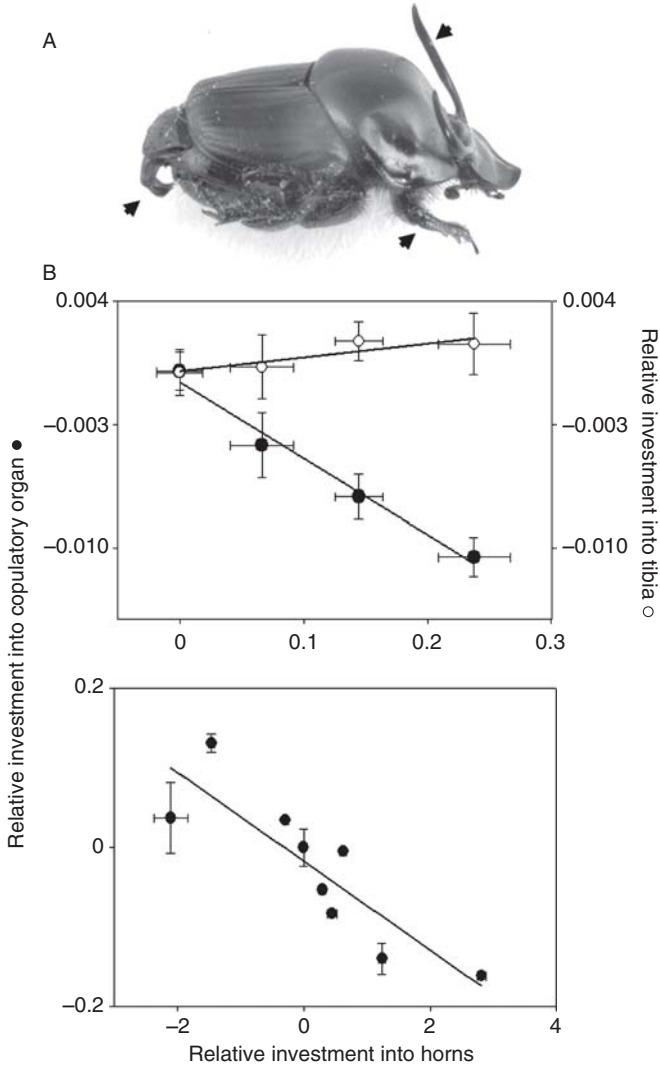


Figure 6.9 Tradeoffs between primary and secondary sexual characters in populations and species of *Onthophagus* beetles. (A) Horned male *Onthophagus taurus*. Arrows highlight horns, copulatory organ, and fore tibia. (B) Relative investment into copulatory organ size (left, ●) and fore tibia size (right, ○) as a function of relative investment into horn size in four different populations of *O. taurus*. Error bars represent one standard error. (C) Relative investment into copulatory organ size as a function of relative investment into horn size in nine different *Onthophagus* species. Data are corrected for differences in body size (modified after Parzer and Moczek, 2008).

implications. First, they suggest that copulatory organ size, a primary sexual trait, may diverge as a byproduct of evolutionary changes occurring in horns, a secondary sexual trait. Second, these findings illustrate that the resulting signatures of antagonistic coevolution are detectable both during microevolutionary divergences between populations operating on a timescale of years, as well as macroevolutionary divergence between species operating on a timescale of tens of millions of years. Third, and most remarkable, given the extreme importance of copulatory organ morphology for reproductive isolation, these findings begin to raise the possibility that secondary sexual trait evolution may promote speciation as a byproduct. If tradeoffs between horns and male copulatory organs are indeed driving speciation in *Onthophagus* this might help explain how this genus, famous for its dramatic diversity in secondary sexual traits, was able to radiate into over 2400 extant species, making it the most speciose genus in the animal kingdom (Arrow, 1951) (Fig. 6.9).



11. CONCLUSIONS

In this chapter, I hope to have shown that horned beetles in general—and the genus *Onthophagus* in particular—offer a rich microcosm in which to explore the mechanisms of evolutionary innovation and diversification. Combining extreme morphological diversity with a rich ecology and natural history as well as developmental and genetic accessibility, research on *Onthophagus* beetles is now beginning to permit an increased integration across levels of biological organization as well as timescales, allowing us to integrate genetic, endocrine and ecological contributions to phenotypic diversity, and to bridge micro- and macroevolutionary perspectives on development. Given the diversity of questions that can be addressed with these organisms and the experimental tools available to researchers, I hope that *Onthophagus* beetles will attract the attention of the next generation of students in evolution and development. It will be up to them to fully realize what we have barely begun to imagine.

ACKNOWLEDGMENTS

Matthew Stansbury provided constructive comments on earlier drafts of this chapter. Research presented here was supported by National Science Foundation grants IOS 0445661 and IOS 0718522.

REFERENCES

- Abzhanov, A., and Kaufman, T. C. (2000). Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. *Dev. Biol.* **227**, 673–689.
- Angelini, D. R., and Kaufman, T. C. (2004). Functional analyses in the hemipteran *Oncopeltus fasciatus* reveal conserved and derived aspects of appendage patterning in insects. *Dev. Biol.* **271**, 306–321.
- Arrow, G. H. (1951). “Horned Beetles.” W. Junk, The Hague, Netherlands.
- Ballerio, A. (1999). Revision of the genus *Pterorthochaetes* first contribution (Coleoptera: Scarabaeoidea: Ceratocanthidae). *Folia Heyrovskyana* **7**, 221–228.
- Balthasar, V. (1963). “Monographie der Scarabaeidae und Aphodiidae der palaearktischen und orientalischen Region (Coleoptera: Lamellicornia). Band 2, Coprinae.” Verlag der tschechoslowakischen Akademie der Wissenschaften, Prag.
- Beebe, W. (1944). The function of secondary sexual characters in two species of Dynastidae (Coleoptera). *Zoologica*. **29**, 53–58.
- Beer mann, A., Jay, D. G., Beerman, R. W., Huelskamp, M., Tautz, D., and Juergens, G. (2001). The *Short antenna* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila Distal-less* protein. *Development* **128**, 287–297.
- Bessa, J., Gebelein, B., Pichaud, F., Casares, F., and Mann, R. S. (2002). Combinatorial control of *Drosophila* eye development by *eyeless*, *homothorax*, and *teashirt*. *Genes Dev.* **16**, 2415–2427.
- Cook, D. (1990). Differences in courtship, mating and postcopulatory behavior between male morphs of the dung beetle *Onthophagus binodis* Thunberg (Coleoptera: Scarabaeidae). *Anim. Behav.* **40**, 428–436.
- Davidson, E. H., and Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science* **311**, 796–800.
- Eberhard, W. G. (1978). Fighting behavior of male *Golofa porteri* beetles (Scarabaeidae: Dynastinae). *Psyche* **83**, 292–298.
- Eberhard, W. G. (1985). “Sexual Selection and Animal Genitalia.” Harvard University Press, Cambridge, MA.
- Emlen, D. J. (1994). Environmental control of horn length dimorphism in the beetle *Onthophagus acuminatus* (Coleoptera, Scarabaeidae). *Proc. R. Soc. Lond. B* **256**, 131–136.
- Emlen, D. J. (1997). Alternative reproductive tactics and male dimorphism in the horned beetle *Onthophagus acuminatus*. *Behav. Ecol. Sociobiol.* **41**, 335–341.
- Emlen, D. J. (2001). Costs and the diversification of exaggerated animal structures. *Science* **291**, 1534–1536.
- Emlen, D. J., and Nijhout, H. F. (1999). Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Insect Physiol.* **45**, 45–53.
- Fristrom, D., and Fristrom, J. W. (1993). The metamorphic development of the adult epidermis. In “The Development of *Drosophila melanogaster*” (M. Bate and A. M. Arias, Eds.), pp. 843–897. Cold Spring Harbor Laboratory Press, New York.
- Hunt, J., and Simmons, L. W. (2001). Status-dependent selection in the dimorphic beetle *Onthophagus taurus*. *Proc. R. Soc. Lond. B* **268**, 2409–2414.
- Inoue, Y., Mito, T., Miyawaki, K., Terasawa, T., Matsushima, K., Shinmyo, Y., Niwa, N., Mito, T., Ohuchi, H., and Noji, S. (2002). Correlation of expression patterns of *homothorax*, *dachshund*, and *Distal-less* with the proximodistal segmentation of the cricket leg bud. *Mech. Dev.* **113**, 141–148.
- Jockusch, E., Nulsen, C., and Nagy, L. M. (2000). Leg development in flies vs. grasshoppers: Differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway. *Development* **127**, 1617–1626.

- Kawano, K. (2002). Character displacement in giant rhinoceros beetles. *Am. Nat.* **159**, 255–271.
- Kojima, T. (2004). The mechanism of *Drosophila* leg development along the proximodistal axis. *Dev. Growth Differ.* **46**, 115–129.
- Lobbia, S., Futahashi, R., and Fujiwara, H. (2007). Modulation of the ecdysteroid-induced cell death by juvenile hormone during pupal wing development of Lepidoptera. *Arch. Insect Biochem. Physiol.* **65**, 152–163.
- Lohmann, I., McGinnis, N., Bodmer, M., and McGinnis, W. (2002). The *Drosophila* Hox gene *deformed* sculpts head morphology via direct regulation of the apoptosis activator *reaper*. *Cell* **23**, 457–466.
- Mittmann, B., and Scholtz, G. (2001). *Distal-less* expression in embryos of *Limulus polyphemus* (Chelicerata, Xiphosura) and *Lepisma saccharina* (Insecta, Zygentoma) suggests a role in the development of mechanoreceptors, chemoreceptors, and the CNS. *Dev. Genes Evol.* **211**, 232–243.
- Mizunuma, T. (1999). “Giant Beetles.” ESI Publishers, Tokyo, Japan.
- Moczek, A. P. (2003). The behavioral ecology of threshold evolution in a polyphenic beetle. *Behav. Ecol.* **14**, 831–854.
- Moczek, A. P. (2005). The evolution and development of novel traits, or how beetles got their horns. *Bioscience* **11**, 935–951.
- Moczek, A. P. (2006a). Integrating micro- and macroevolution of development through the study of horned beetles. *Heredity* **97**, 168–178.
- Moczek, A. P. (2006b). Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *Am. Nat.* **168**, 711–729.
- Moczek, A. P. (2007). Pupal remodeling and the evolution and development of alternative male morphologies in horned beetles. *BMC Evol. Biol.* **7**, 151.
- Moczek, A. P., and Emlen, D. J. (1999). Proximate determination of male horn dimorphism in the beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Evol. Biol.* **12**, 27–37.
- Moczek, A. P., and Emlen, D. J. (2000). Male horn dimorphism in the scarab beetle *Onthophagus taurus*: Do alternative tactics favor alternative phenotypes? *Anim. Behav.* **59**, 459–466.
- Moczek, A. P., and Nagy, L. M. (2005). Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. *Evol. Dev.* **7**, 175–185.
- Moczek, A. P., and Nijhout, H. F. (2002). Developmental mechanisms of threshold evolution in a polyphenic beetle. *Evol. Dev.* **4**, 252–264.
- Moczek, A. P., and Nijhout, H. F. (2003). Rapid evolution of a polyphenic threshold. *Evol. Dev.* **5**, 259–268.
- Moczek, A. P., and Nijhout, H. F. (2004). Trade-offs during the development of primary and secondary sexual traits in a horned beetle. *Am. Nat.* **163**, 184–191.
- Moczek, A. P., and Rose, D. J. (2009). Differential recruitment of limb patterning genes during development and diversification of beetle horns (unpublished data).
- Moczek, A. P., Hunt, J., Emlen, D. J., and Simmons, L. W. (2002). Threshold evolution in exotic populations of a polyphenic beetle. *Evol. Ecol. Res.* **4**, 587–601.
- Moczek, A. P., Rose, D., Sewell, W., and Kesselring, B. R. (2006). Conservation, innovation, and the evolution of horned beetle diversity. *Dev. Genes Evol.* **216**, 655–665.
- Nagy, L. M., and Williams, T. A. (2001). Comparative limb development as a tool for understanding the evolutionary diversification of limbs in arthropods: Challenging the modularity paradigm. In “The Character Concept in Evolutionary Biology” (G. Wagner, Ed.), pp. 457–490. Academic Press, San Diego, CA.
- Nijhout, H. F., and Emlen, D. J. (1998). Competition among body parts in the development and evolution of insect morphology. *Proc. Natl. Acad. Sci. USA* **95**, 3685–3689.
- Oliver, R. H., Albury, A. N., and Mousseau, T. A. (2007). Programmed cell death in flight muscle histolysis of the house cricket. *J. Insect Physiol.* **53**, 30–39.

- Palmer, T. J. (1978). A horned beetle which fights. *Nature* **274**, 583–584.
- Parzer, H. F., Moczek, A. P. (2008). Rapid antagonistic coevolution between primary and secondary sexual characters in horned beetles. *Evol.* **62**, 2423–2428.
- Paulian, R. (1935). Le polymorphisme des males de coléoptères. In “Exposés de biométrie et statistique biologique IV” (G. Tessier, Ed.), pp. 1–33. Actualités scientifiques et industrielles 255. Hermann and Cie, Paris, France.
- Paulian, R. (1945). “Coléoptère Scarabéides de l’Indochine. Première partie.” Faune de l’Empire Français III, Paris, France.
- Potten, C., and Wilson, J. (2004). “Apoptosis: The Life and Death of Cells.” Cambridge University Press, Cambridge, MA.
- Prpic, N. M., and Tautz, D. (2003). The expression of the proximo-distal patterning genes *Distal-less* and *dachshund* in the appendages of *Glomeris marginata* (Myriapoda, Diplopoda) suggest a special role of these genes in patterning head appendages. *Dev. Biol.* **260**, 97–112.
- Prpic, N. M., Wigand, B., Damen, W. G., and Klingler, M. (2001). Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. *Dev. Genes Evol.* **211**, 467–477.
- Raff, R. (1996). “The Shape of Life: Genes Development, and the Evolution of Animal Form.” University of Chicago Press, Chicago, IL.
- Ryoo, H. D., Marty, T., Casares, F., Affolter, M., and Mann, R. S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* **126**, 5137–5148.
- Schoppmeier, M., and Damen, W. G. M. (2001). Double-stranded RNA interference in the spider *Cupiennius salei*: The role of *Distal-less* is evolutionarily conserved in arthropod appendage formation. *Dev. Genes Evol.* **211**, 76–82.
- Shelby, J. A., Madewell, R., and Moczek, A. P. (2007). Juvenile hormone mediates sexual dimorphism in horned beetles. *J. Exp. Zool. B* **308**, 417–427.
- Shepherd, B. L., Prange, H. D., and Moczek, A. P. (2008). Some like it hot: Body and weapon size affect thermoregulation in horned beetles. *J. Insect Physiol.* **54**, 604–611.
- Simmons, L. W., and Emlen, D. J. (2006). Evolutionary trade-off between weapons and testes. *Proc. Natl. Acad. Sci. USA* **103**, 16346–16351.
- Simmons, L. W., Tomkins, J. L., and Hunt, J. (1999). Sperm competition games played by dimorphic male beetles. *Proc. R. Soc. Lond. B* **266**, 145–150.
- Simmons, L. W., Emlen, D. J., and Tomkins, J. L. (2007). Sperm competition games between sneaks and guards: A comparative analysis using dimorphic male beetles. *Evolution* **61**, 2684–2692.
- Siva-Jothy, M. T. (1987). Mate securing tactics and the cost of fighting in the Japanese horned beetle, *Allomyrina dichotoma* L. (Scarabaeidae). *J. Ethol.* **5**, 165–172.
- Suzuki, Y., and Palopoli, M. F. (2001). Evolution of insect abdominal appendages: Are prolegs homologous or convergent traits? *Dev. Genes Evol.* **211**, 486–492.
- Svácha, P. (1992). What are and what are not imaginal discs: Reevaluation of some basic concepts (Insecta, Holometabola). *Dev. Biol.* **154**, 101–117.
- West-Eberhard, M. J. (2003). “Developmental Plasticity and Evolution.” Oxford University Press, New York.
- Yao, L. C., Liaw, G. J., Pai, C. Y., and Sun, Y. H. (1999). A common mechanism for antenna-to-leg transformation in *Drosophila*: Suppression of homothorax transcription by four HOM-C genes. *Dev. Biol.* **211**, 268–276.
- Ziani, S. (1994). Un interessante caso di teraologia simmetrica in *Onthophagus (Paleonthophagus) fracticornis* (Coleoptera, Scarabaeidae). *Boll. Ass. Romana Entomol.* **49**, 165–167.