

Emerging model systems in evo-devo: horned beetles and the origins of diversity

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SUMMARY Horned beetles and beetle horns are emerging as a model system suited to address fundamental questions in evolutionary developmental biology. Here we briefly review the biology of horned beetles and highlight the unusual opportunities they provide for evo-devo research. We then summarize recent advances in the development of new

approaches and techniques that are now available to scientists interested in working with these organisms. We end by discussing ways to implement and combine these new approaches to explore new frontiers in evo-devo research previously unavailable to researchers working outside traditional model organisms.

INTRODUCTION

The horns of beetles have fascinated biologists since Darwin's time, in part for some of the same reasons that now account for their growing attractiveness as model systems in evolutionary developmental biology (Wallace 1869; Darwin 1871). For starters, beetle horns are major structures often exceeding the weight of legs or wings. There is nothing subtle about beetle horns; instead, they frequently dominate and define the physical appearance of their bearers. Furthermore, they are extraordinarily diverse, protruding from various locations on the head, prothorax, or both (Arrow 1951). Differences in horn phenotypes arise for both genetic and environmental reasons. For example, although genetic differences account for diversity in horn expression between species, conspecific populations, and sexes within species (females typically lack horns), nutritional environments determine horn development within sexes, including the expression of discretely different alternative male morphologies (Emlen 1994; Moczek 1998; Moczek and Nijhout 2002, 2003). Most importantly, however, the horns of beetles are attractive to evolutionary biologists because they are unique structures, lacking obvious homology to other structures in insects and non-insect arthropods. Beetle horns are therefore an example of an evolutionary novelty, which has undergone dramatic diversification within a relatively narrow phylogenetic context. Together, this provides researchers with the opportunity to address several fundamental and largely unresolved questions in evo-devo, including: how do novel features arise and diversify in nature? How do ecological, genetic, and develop-

mental mechanisms interact in the genesis of novelty and diversity? What is the relationship between micro- and macroevolution of development?

A BRIEF NATURAL HISTORY OF BEETLE HORNS

Several thousand species of beetles express horns (Balthasar 1964; Matthews 1972), and even though only a relatively small number of species have been studied in any detail, there is clear evidence that horns function as weapons in male competition over mates. Depending on species, habitat, horn number, and location, beetles use their horns to block, prod, shove, whack, dislodge, flip, or stab rival males (e.g., Eberhard 1978, 1979; Rasmussen 1984; Emlen 1997; Moczek and Emlen 2000; Eberhard et al. 2000), and experiments have quantified the fitness consequences of horn possession in selected species (Hunt and Simmons 2001). In species with alternative horned and hornless male morphologies, males that lack horns avoid aggressive encounters and instead engage in nonaggressive sneaking behaviors to circumvent horned rivals (Moczek and Emlen 1999). Horns can be found in at least six beetle families, but are by far the most diverse and extreme in the family Scarabaeidae, taxonomic home to the spectacular subfamily Dynastinae including the SE-Asian genus *Chalcosoma* admired by Darwin for their extreme size, armature, and degree of sexual dimorphism, and the subfamily Scarabaeinae, known for its diversity of dung beetles, including the genus *Onthophagus*, which, over the past decade, has emerged as a particularly important microcosmos of evolutionary innovation and diversification. *Onthophagus* represents, with an

estimated 2400 species, one of the most speciose genera in the animal kingdom, and the dramatic diversity in horn expression observed among even closely related *Onthophagus* species is unparalleled (Balthasar 1964; Matthews 1972). *Onthophagus* is also home to some of the most extreme male dimorphisms, generating alternative horned and hornless morphs so distinct that they have occasionally been described as separate species (Paulian 1935). At the same time, however, most *Onthophagus* are small and similar in size to a coffee bean or peanut with a generation time of about 5–7 weeks (e.g., compared with the giant *Chalcosoma* or *Dynastes* whose larvae require several years to mature). *Onthophagus* species can be found on every continent, and several of the most interesting species, such as *Onthophagus taurus*, can now be found across multiple continents due to several deliberate or accidental introductions to non-native habitats. At least six diverse *Onthophagus* species have so far been reared sustainably in captivity for multiple generations with relative ease. Two recent molecular phylogenies of a small number of species, including these six, have given us a first glimpse of the evolutionary history of this genus (Villalba et al. 2002; Emlen et al. 2005a, b). Together, this now permits us for the first time to bring horned beetles to a developmental geneticist's lab bench, and to explore the developmental basis of beetle horns in an evolutionary context.

DEVELOPMENT OF HORNS AND HORN DIVERSITY

Histological analyses show that horns originate during the prepupal stage of late larval development as epidermal out-buddings of selected regions of the larval epithelium. Following an earlier period of apolysis, or detachment of the larval epidermis from the cuticle, these selected regions now undergo more or less dramatic cell proliferation, which throws the resulting new tissue into folds as it remains confined underneath the larval cuticle. Upon pupation, the animal then sheds the old larval cuticle, allowing the pupal precursors of adult structures to unfold and expand (Moczek and Nagy 2005). The precursors of adult horns originate during this prepupal growth phase similar to the precursors of adult legs, mouthparts, wings, or antennae of many insect orders (Svacha 1992). The only deviation from this pattern occurs in the higher flies such as *Drosophila*, and the wings of Hymenoptera, Lepidoptera, and some Coleoptera, where appendages develop from early developing imaginal discs (sensu Svacha 1992). These observations provided the first hint that the development of horns may be mediated at least in part by some of the same developmental mechanisms that regulate the expression of more typical insect appendages such as legs or mouthparts. At the same time, it has become clear that there is more to horns than growth. During the pupal stage, and

thus following the prepupal growth period, at least some horn types are extensively remodeled, in extreme cases causing fully horned pupae to molt into entirely hornless adults (Moczek et al. 2006a). This behavior is in marked contrast to that of all other pupal appendages that are never fully resorbed during pupal development and instead sculpted into their final adult shape via localized-programmed cell death (Lohmann et al. 2002; Cullen and McCall 2004). In contrast, pupal remodeling of horns including resorption is not only widespread but at the same time evolutionary labile, often restricted to one sex or pronounced to different degrees in different species (Moczek 2006a, b; Moczek et al. 2006a). Combined, these observations suggest that horn expression and diversity are the products of at least two developmentally dissociated processes: a prepupal growth phase late in larval development, followed by a pupal remodeling phase just before the final, adult molt. Importantly, these observations also form the basis for the development of hypotheses regarding the genetic underpinnings of growth and remodeling, and their respective roles in the diversification of horned beetles.

ANALYSIS OF CANDIDATE GENES THROUGH EXPRESSION PROFILING

Studies on appendage formation in a variety of insects have revealed that despite some major differences in the mode of appendage formation across different groups of insects, there remain many similarities in the underlying patterning mechanisms (Panganiban et al. 1994; Lecuit et al. 1997; Kojima 2004; Angelini and Kaufman 2005). This also holds true for appendage development in a close cousin of *Onthophagus*, the flour beetle genus *Tribolium*, where many detailed and elegant studies on appendage development prepared the ground for a comparative approach in the context of *Onthophagus* horn development (e.g., Brown et al. 1999; Bucher et al. 2002; Beermann and Schroder 2004; Tomoyasu and Denell 2004). A particularly striking similarity in appendage patterning across insect orders involves the formation of the proximo-distal (*p/d*) axis, which appears to rely on the expression and interaction of four conserved transcription factors: *Distal-less* (*Dll*), *dachshund* (*dac*), *homothorax* (*hth*), and *nuclear-extradenticle* (*n-exd*). For example, in the *Drosophila* leg *Dll* expression is confined to the center of the leg imaginal disc, which will eventually give rise to the distal regions of the adult appendage, while progressively more peripheral disc regions, characterized by *dac* and *hth/n-exd* expression, form progressively more proximal appendage regions once the imaginal disc telescopes outward to form the adult appendage (Abu-Shaar and Mann 1998; Wu and Cohen 1999). *Tribolium* beetles (Beermann et al. 2001; Prpic et al. 2001), but also *Gryllus* crickets (Inoue et al. 2002), and *Schistocerca* grasshoppers

(Jockusch et al. 2000, 2004) share a similar arrangement of expression patterns along the *p/d*-axis of developing appendages, and *Dll* activity had been shown to be functionally required for distal leg formation in *Tribolium* beetles as well as spiders (Beermann et al. 2001; Schoppmeier and Damen 2001). Given the congruence of *p/d* patterning across insects and appendage types, *Dll*, *dac*, *hth*, and *n-exd* represent promising candidate genes for the patterning of horns. Comparative gene expression studies using pangenic antibodies and in situ hybridization have since confirmed a potential *p/d* patterning function of three of the four genes (Moczek and Nagy 2005; Moczek et al. 2006b). *Dll* is expressed during horn development in those epidermal cells that will later form the distal region of the horn, whereas *hth* and *n-exd* expression is largely confined to proximal horn regions. In contrast, *dac* expression shows no obvious subdivision and instead is ubiquitous in all horn regions including outside its predicted medial domain, consistent with a lack or loss of patterning function during horn development. Combined, these data suggest that the origin of beetle horns involved at least the partial co-option of traditional appendage patterning elements into a novel morphological and behavioral context: the evolution of a novel appendage type used as a weapon in male competition. More detailed analyses of these and additional candidate genes in pathways relevant to the formation, growth, patterning, and remodeling of horns, such as *egf*- and insulin-signaling and the regulation of programmed cell death, are currently ongoing and will, over time, increase our understanding of the complexity of the developmental genetic processes associated with horn development and evolution (Emlen et al. 2006). Recent methodological advances now permit researchers to move even one step further and to examine the genetic basis of horn development and diversity through comparative gene function analysis.

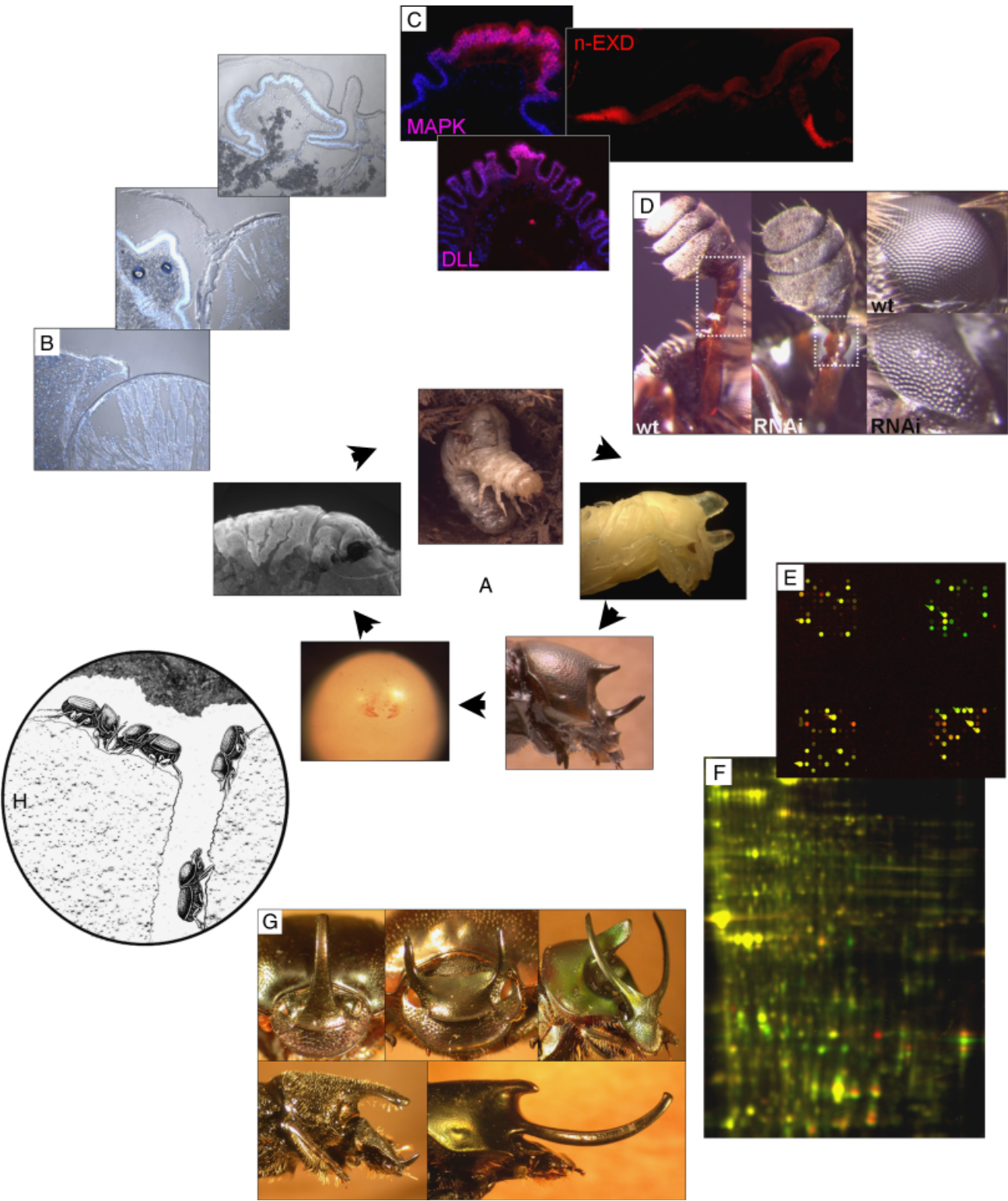
FUNCTIONAL ANALYSIS THROUGH LARVAL RNA INTERFERENCE

RNA interference, the experimental gene-specific depletion of transcription levels via exogenous application of *dsRNA* fragments derived from a selected target gene, has revolutionized evolutionary developmental genetics due to its applicability across a wide range of organisms (Novina and Sharp 2004). Not surprisingly, RNAi-mediated gene knockdown has since been used in a range of arthropod taxa to examine gene function in a variety of developmental contexts, including appendage patterning (Hughes and Kaufman 2000; Schoppmeier and Damen 2001; Angelini and Kaufman 2005; Herke et al. 2005). In *Tribolium* beetles, RNAi-mediated knockdown of patterning genes, including maternal RNAi as well as injections during embryonic and larval development,

have worked particularly well and often with spectacular results (Brown et al. 1999; Bucher et al. 2002; Beermann and Schroder 2004; Tomoyasu and Denell 2004; Tomoyasu et al. 2005; Ober and Jockusch 2006). Horned beetles are now beginning to follow in *Tribolium*'s footsteps, and recent studies have successfully used larval RNAi to knock down expression levels to examine the function of a series of developmental patterning genes in *Onthophagus*. Experiments have initially focused on genes with well-described depletion-of-function consequences in *Drosophila* and *Tribolium*, and in each case RNAi has yielded matching and clearly interpretable phenotypic effects in *Onthophagus* (Moczek et al. in prep.; see Fig. 1 e.g.). Successes with RNAi-mediated transcript depletion in other horned beetle taxa have since also been reported from other labs (D. J. Emlen and L. Corley, personal communication). Experiments are now underway to examine candidate genes in pathways relevant to the formation, growth, and patterning of horns across species with diverse horn phenotypes. Ultimately, the existence of working larval RNAi protocols now equips researchers with the ability to examine the significance of practically any genes of interest relevant to horn development and evolution as well as other phenotypes.

FROM CANDIDATE GENES TO A GENOMIC PERSPECTIVE—MICROARRAYS AND DIFFERENTIAL GEL ELECTROPHORESIS (DIGE)-ELECTROPHORESIS

A candidate gene approach is ultimately limited by the experimental data available from other organisms. In the case of *Onthophagus* beetles, this includes primarily experimental studies on *Tribolium* beetles, *Drosophila*, or the milkweed bug *Oncopeltus* (reviewed in Angelini and Kaufman 2005). Such an approach is, almost by definition, unable to discover new genes and their function, which instead requires an approach that permits researchers to examine the sum total of all genes associated with the expression of a given phenotype or a particular developmental stage. Two such approaches are now being developed for *Onthophagus* beetles. The first involves the development of microarrays with several thousand expressed sequence tags derived from normalized larval and pupal cDNA libraries for comparative transcriptional profiling on a genome-wide scale (Kijimoto et al. unpublished data). The second approach involves two-dimensional (2D) DIGE, where multiple protein samples are labeled and proteins are then separated according to isoelectric focusing point and size on a 2D gel (Unlu et al. 1997; Yerushalmi and Moczek unpublished data). Proteins shared by samples co-migrate, whereas differentially expressed or posttranscriptionally modified proteins occupy unique spots on the gel. Such proteins can then be extracted, their mass and their amino acid



sequence can be determined via mass-spectrophotometry and aligned against available databases. Both methods are currently used to characterize transcript and protein profiles across sexes, but will ultimately be expanded to include multiple species, alternative morphs within sexes, and even different tissue regions within morphs.

CURRENT FRONTIERS IN HORNED BEETLE EVO-DEVO

With the newly available approaches summarized above, researchers interested in working with horned beetles are now able to address questions in evolutionary developmental biology in a manner previously impossible for most non-model organisms. Target gene, genomic, and gene function analysis permit connecting phenotype to genotype in a thorough and rigorous manner, and exploring the genes and pathways whose redeployment mediated the origin and diversification of horns, as well as any other phenotype of interest. At the same time, these approaches also allow us to explore aspects of developmental interaction and integration across levels of biological organization. With respect to horns, for example, we can now ask how genetic and endocrine regulators such as juvenile hormone or ecdysteroids interact during horn development, and whether and how these interactions have changed during the radiation of horned beetles. Lastly, by addressing these questions comparatively across morphs, sexes, populations, species, and genera, we can begin integrating micro- and macroevolutionary perspectives of development. For example, we can ask whether signatures of macroevolutionary events normally characteristic of higher-level taxonomic divergences can be observed among natural populations or closely related species. The past 10 years have already witnessed an impressive increase in interest and understanding of horned beetle evolution and development and we are optimistic that over the next 10 years, horned beetles will be able to provide many valuable and probably surprising insights

into the mechanisms of innovation and diversification in the natural world.

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Fig. 1. Horned beetles in the genus *Onthophagus* are evolutionarily interesting and developmentally accessible. (A) Typical life stages of *Onthophagus* from egg and embryo through three larval stages, the pupa, and adult. (B–H) Examples of approaches developed to investigate and integrate diverse levels of biological organization of horned beetle genetics, development, ecology, and evolution. (B) Comparative histological approaches provide the foundation for the development of hypotheses regarding the developmental regulation of phenotypic diversity in horned beetles. (C) Comparative gene expression studies help to further refine these hypothesis, which can subsequently be tested more rigorously via RNAi-mediated gene function analysis (D). Examples shown here are results from larval RNAi-mediated transcript depletion of the patterning gene *dachshund*, which is known to pattern medial appendage identity as in the beetle antenna, as well as eye formation and differentiation. (E) Microarray and (F) 2D differential gel electrophoresis (DIGE) approaches permit transition from candidate gene to genomic and proteomic perspectives on the origin and diversification of horned beetles and beetle horns. For example, the two-dimensional gel shown here contrasts the proteome expressed in the brains of alternative horned (red) and hornless (green) male morphs. Protein expression shared across morphs appears in yellow. (G, H) Integrating these diverse approaches into the ecological and social context within which horned beetles function provides the rare opportunity to explore the population biology of developmental evolution and to begin integrating micro- and macroevolutionary perspectives on development.

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