Diverse developmental mechanisms contribute to different levels of diversity in horned beetles

Armin P. Moczek\textsuperscript{a,*} and Lisa M. Nagy\textsuperscript{b}

\textsuperscript{a}Department of Biology, Indiana University, Bloomington, IN, USA
\textsuperscript{b}Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA

*Author for correspondence (email: armin@indiana.edu)

SUMMARY An ongoing challenge to evolutionary developmental biology is to understand how developmental evolution on the level of populations and closely related species relates to macroevolutionary transformations and the origin of morphological novelties. Here we explore the developmental basis of beetle horns, a morphological novelty that exhibits remarkable diversity on a variety of levels. In this study, we examined two congeneric \textit{Onthophagus} species in which males develop into alternative horned and hornless morphs and different sexes express marked sexual dimorphism. In addition, both species differ in the body region (head vs. thorax) that develops the horn. Using a comparative morphological approach we show that prepupal growth of horn primordia during late larval development, as well as reabsorption of horn primordia during the pupal stage, contribute to horn expression in adults. We also show that variable combinations of both mechanisms are employed during development to modify horn expression of different horns in the same individual, the same horn in different sexes, and different horns in different species. We then examine expression patterns of two transcription factors, \textit{Distal-less} (\textit{Dll}) and \textit{aristaless} (\textit{al}), in the context of prepupal horn growth in alternative male morphs and sexual dimorphisms in the same two species. Expression patterns are qualitatively consistent with the hypothesis that both transcription factors function in the context of horn development similar to their known roles in patterning a wide variety of arthropod appendages. Our results suggest that the origin of morphological novelties, such as beetle horns, rests, at least in part, on the redeployment of already existing developmental mechanisms, such as appendage patterning processes. Our results also suggest, however, that little to no phylogenetic distance is needed for the evolution of very different modifier mechanisms that allow for substantial modulation of trait expression at different time points during development in different species, sexes, or tissue regions of the same individual. We discuss the implications of our results for our understanding of the evolution of horned beetle diversity and the origin and diversification of morphological novelties.

INTRODUCTION

A fundamental goal of evolutionary biology is to understand the mechanisms by which novel phenotypes originate and diversify (West-Eberhard 2003; Minelli 2003). Population-genetic studies have shown that many developmental processes can respond to artificial or natural selection and can mediate substantial modification of existing phenotypic traits (e.g., Hazel and West 1982; Semlitsch and Wilbur 1989; Zera and Zhang 1995; Emlen 1996; Beladade and Brakefield 2002; Moczek and Nijhout 2002a; Zijlstra et al. 2004), but have generally provided little insights into how novel traits may originate in the first place. Comparative studies of widely divergent taxa have provided a different perspective and have emphasized the roles of redeployment and recruitment of already existing developmental and genetic processes into different developmental contexts as a major avenue of organismal innovation (reviewed in True and Carroll 2002). This perspective has been valuable for understanding how a limited set of developmental processes can accommodate and generate a wide range of highly diverse phenotypes, but has been largely unable to relate macroevolutionary innovation to the more subtle and quantitative developmental evolution that occurs in natural populations. As a consequence, micro- and macroevolutionary perspectives on development remain largely disconnected, and we have yet to learn if and how microevolutionary changes in developmental processes are necessary or sufficient for the origin of major evolutionary novelties (Raff 1996; Schlichting and Pigliucci 1998; Gilbert 2001; West-Eberhard 2003).

Polyphenic organisms, that is, organisms in which individual genotypes are able to develop into two or more discretely different phenotypes as a function of environmental factors, provide a possibly valuable opportunity to address these issues within narrow taxonomic boundaries (Gilbert 2001). Polyphenic development involves the facultative
expression of morphological structures that are typically considered evolutionary novelties (West-Eberhard 2003), such as the facultative expression of wings in ants and termites (Abouheif and Wray 2002), spot patterns in seasonally polyphenic butterflies (Brakefield et al. 1996; Nijhout and Wheeler 1996), or alternative terrestrial and aquatic life stages in salamanders (Semlitsch et al. 1990). Furthermore, while environmental factors may regulate a given trait in the context of a polyphenism, the same trait in the same species may at the same time be regulated by genetic factors in the context of sexual dimorphisms or evolved differences in phenotype expression between populations and species (Tauber and Tauber 1970, 1972, 1982; Hazel and West 1982; Denno et al. 1996; Moczek and Nijhout 2003). Polyphenic organisms therefore can provide important insights into the developmental processes that generate morphological novelties, the interplay between genetic and environmental factors in instructing these processes, as well as the means by which these processes have diversified on different levels. In this paper we begin to explore the developmental regulation of beetle horns, a morphological novelty with remarkable intra- and interspecific diversity.

Beetle horns are among the most diverse exaggerated secondary sexual traits in the animal kingdom (Arrow 1951; Balthasar 1965; Matthews 1972). Several thousand species of beetles develop horns of some kind, and horn expression varies dramatically on a variety of scales. Males in many species express discrete, alternative hornless, and horned morphologies separated by critical body size thresholds with intermediates being rare or absent. In such species each individual male has the ability to express either morph, and which morph a given male develops into depends largely on larval nutrition (Moczek 1998). While male morph determination is thus largely determined by environmental conditions, conspecific populations can differ dramatically and heritably in certain aspects of the environment-sensitive switch mechanism, such as the exact location of the threshold body size (Moczek 2003). At the same time horns are expressed in a sex-specific manner. Females typically lack horns completely or express rudimentary horns compared with their male counterparts (Arrow 1951). Lastly, even closely related species can differ dramatically in the number of horns and the body region that produces them. In the genus Onthophagus horn phenotypes range from single or paired horns produced on either the head or thorax to combinations of variable numbers of head and thoracic horns produced by the same animal (Arrow 1951; Balthasar 1965; Matthews 1972). Even though horns are widespread and diverse in many groups of beetles, they have no obvious homologue in other arthropod structures (Moczek 2005).

Beetle horns originate from selected regions of the larval epidermis, which undergo rapid growth during the prepupal stage of late larval development (Emlen and Nijhout 1999). Even though beetle horns lack joints, muscles, and nervous tissue, their development thus appears at least in some aspects similar to that of regular insect appendages. Numerous studies have shown that though insect appendages such as legs, mouthparts, or antennae are remarkably diverse structures, they share a largely conserved network of patterning genes required for correct differentiation (reviewed in Panganiban et al. 1997; Nagy and Williams 2001; Kojima 2004). In Drosophila, appendages such as legs, antennae, or genitalia develop from imaginal discs; monolayered groups of imaginal cells set aside from larval tissues during embryogenesis. During larval development the concentration-dependent combined action of two diffusible morphogens, wingless (wg) and decapentaplegic (Dpp) subdivides imaginal discs 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 disc, characterized by Dll expression, eventually gives rise to the distal region of the adult appendage, while progressively more peripheral disc regions, characterized by dac and hth expression, form progressively more proximal appendage regions once the imaginal disc telescopes outwards to form the adult appendage (Lecuit and Cohen 1997; Abu-Shaar and Mann 1998; Wu and Cohen 1999; Kojima 2004). In many other arthropods adult appendages develop not from imaginal discs but via the outbudding of selected epidermal regions during larval development (e.g., Fristrom and Fristrom 1993; Nagy and Williams 2001; Prpic et al. 2003). Despite these fundamental differences in the morphogenesis of appendages there remain many similarities in the molecular mechanisms used in patterning diverse appendage. 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 non-insect arthropods (Abzhanov and Kaufman 2000; Jockusch et al. 2000; Mittmann and Scholtz 2001; Suzuki and Palopoli 2001; Inoue et al. 2002; Prpic et al. 2003; Prpic and Tautz 2003), and Dll activity has been shown to be functionally required for distal leg formation in beetles and spiders (Beermann et al. 2001; Schoppmeyer and Damen 2001).

In Drosophila legs WG/DPP interactions also instruct the expression of an additional tier of patterning elements, epidermal growth factor receptors (EGFR) and their ligands. In Drosophila EGFR expression forms steep gradients from the future apical tip to the proximal regions of the developing appendage (Barolo and Posakony 2002; Campbell 2002; Galindo et al. 2002) and graded EGFR expression regulates a suite of additional transcription factors such as aristaless (al), BarH1/BarH2 (bar), bric-a-brac (bab) and rotund (rm) (Campbell 2002; Kojima 2004). While the role of EGFR has been little studied outside Drosophila, their targets have been investigated in other arthropods. In Drosophila, aristaless patterns tarsal segment formation in legs (Campbell 2002) and the formation of the arista on the antenna (Schneitz et al.
1993). Its ortholog in crickets is expressed in the distal portions of developing legs, mouthparts, and antennae as well as in the cerci (Miyawaki et al. 2002). Given the congruence in patterning mechanisms across a wide range of appendage types and developmental modes, we therefore hypothesized that horn development in beetles may be patterned by some of the same genes that pattern regular appendages in insects.

Here we explore the dynamics, timing, and genetic regulation of horn development in two congeneric species of horn polyphenic and sexually dimorphic beetle species. Using a comparative morphological approach we show that different mechanisms, operating at different developmental stages and time scales, affect if and to what extent horns develop in adult beetles. We then examine expression patterns of two transcription factors, Distal-less (Dll) and aristaless (al), in the context of prepupal horn growth in alternative male morphs and sexual dimorphisms in the same two species. We focus our investigation on two hypotheses: (1) DLL and AL function in a conserved manner during horn development similar to their role in the development of other insect appendages such as legs. (2) Differential expression of Dll and al regulate the extent of horn expression in alternative male morphs and females. Based on these hypotheses we predict DLL protein to be present in the distal regions, and AL expression to be restricted to the extreme tips, of developing horns. At the same time, we predict expression domains of either transcription factor to be greatly reduced or absent in minor males and hornless females of both species. We discuss our findings in the context of the origin of morphological novelties and the mechanisms of diversification of secondary sexual traits in beetles.

MATERIAL AND METHODS

Species choice
To explore the development of beetle horns we studied two horn polyphenic and sexually dimorphic Onthophagus species that differ markedly in the location of horn development (Fig. 1, A and B). Large adult male Onthophagus taurus (Fig. 1A) develop a pair of long curved horns on their heads, whereas small males only develop rudimentary horns in the same location. In place of head horns females of all adult sizes develop a broad, minor ridge across the head. Adult O. taurus never develop a horn on their pronotum. In the second species Onthophagus nigriventris (Fig. 1B), large males develop a single, long, and curved pronotal (thoracic) horn. In place of a horn, small adult males of this species develop a prominent pointy projection of the pronotum, whereas females of all adult sizes develop a broad, minor ridge. Adult O. nigriventris never develop a horn on their head (Fig. 1B).

Beetle rearing
Beetle laboratory colonies were derived from animals collected from pastures near Durham, North Carolina (O. taurus) and near Waimea, Hawaii (O. nigriventris). Both laboratory colonies were kept in an insectary at University of Arizona at 25°C under a 16:8 light:dark cycle. Beetles were bred in plastic containers (35-cm tall, 20-cm 2) filled 3/4 with a moist sand/soil mixture. Five pairs of beetles were added per container and provided with 1/240.5 liter of homogenized cow dung. Eight days later beetles were removed and brood balls were collected. By this time most larvae had already hatched and passed through the first instar. The following day, a subset of brood balls was carefully opened and second-instar larvae were transferred into artificial growth containers and provided with an unlimited food supply for the remainder of the larval stage (for details on containers and diet see Shafiei et al. 2001). Larval growth containers were kept in a constant temperature incubator at 25°C and in complete darkness except for a brief daily examination. Using this method we followed 480 O. taurus and 300 O. nigriventris through development from late second larval instar to adulthood. Key morphological changes were documented using a digital camera (Nikon Coolpix 995, Melville, NY, USA) mounted onto a stereo microscope (Leica S8APO, Wetzlar, Germany).

Predicting sex and male adult phenotype
Larvae were sexed around day 8 (O. taurus) and day 10 (O. nigriventris) of the third instar based on the presence/absence of genital imaginal disks (present in males only, see Moczek and Nijhout 2002b). Larvae were then allowed to grow until the onset of the prepupal stage. At this point larvae cease to feed, purge their
gut, undergo a color change and become generally passive. Larvae were weighed at this transition and the weight used to predict male horn phenotype. O. taurus males weighing $<0.11$ g as first day prepupae developed into hornless, male pupae ($n = 54$), while males weighing $>0.125$ g developed horned major males without exception ($n = 25$). Male first day prepupa weighing $0.11-0.125$ g could not be scored with accuracy and thus were excluded from the study. For O. nigrientris the critical weights were $<0.21$ g (hornless, $n = 24$) and $>0.26$ g (horned, $n = 18$), and males weighing $0.21-0.26$ g were excluded from the study. During the second day of the prepupal stage, individuals assume a characteristic posture, including a newly shrunken abdomen and a largely straight body. First and second day prepupae can therefore be easily distinguished. For the present study we used 1-day-old prepupae at the transition to the second-day prepupal stage. At this stage horn primordia have undergone most of their growth and can be clearly distinguished. For the present study we used 1-day-old prepupae at the transition to the second-day prepupal stage. At this stage horn primordia have undergone most of their growth and can be clearly recognized in sections while the epidermis has not yet produced the future pupal cuticle, which otherwise interferes with antibody staining procedures.

Fixation and sectioning
We removed the abdomens of prepupa with a razor blade behind the third thoracic segment. Heads and thorax were dropped immediately into equal amounts of 4% formaldehyde in phosphate-buffered saline and heptane for 60 min at room temperature (RT). Fixed tissue was stored in 100% methanol at $-20^\circ$C until use. Prior to sectioning prepupae were transferred to a 30% sucrose solution in phosphate-buffered saline+0.1% Triton X-100 and allowed to equilibrate for 24 h at 4°C. Prepupae were then freeze-mounted in OCT Embedding Medium (Electron Microscopy Sciences, West Chester, PA, USA) and cryosectioned at $-25^\circ$C into 14-20-μm thick sections using a cryostat (Microm, Heidelberg, Germany). Sections were placed on microscope slides and stored at $-20^\circ$C until further use.

Immunohistochemistry
We investigated the distribution of DLL and AL proteins in the horn precursors of 1-day-old prepupae of both Onthophagus species by immunohistochemistry using antibodies previously shown to recognize DLL and AL antigens in diverse species (DLL: Panginban et al. 1995; other refs; gift from G. Boekhoff-Falk; AL; Campbell ref; grasshopper ref; gift from G. Campbell). Microscope slides with sections were mounted in RT and slide edges were marked with a Pap Pen (Electron Microscopy Sciences; West Chester, PA, USA). Sections were washed 3 times with phosphate-buffered saline+0.1% Triton X-100 (PBT) for 10 min per wash. Sections were then incubated with a 2% bovine serum albumin solution (Roche, Indianapolis, IN, USA) in PBT for 5 h at RT, followed by two quick washes with PBT. Sections were then incubated in primary antibody (DLL 1:100; AL 1:500) overnight at 4°C. The next morning the primary antibody solution was removed and sections were washed at RT 3 times quickly with PBT, followed by six washes in 10-min intervals. Sections were then incubated in anti-rabbit (DLL) or anti-rat (AL) secondary CY3-conjugated antibody, diluted 1:200 in blocking serum for 2 h. Sections were then washed quickly 3 times in PBT followed by three washes in 10-min intervals. Sections were counterstained with 4’,6-Diamidino-2-phenylindole (DAPI; 1:1000 solution in PBT) (Hoechst, Mannheim, Germany) for 10 min, washed 3 × 1 × for 10 min with PBT and mounted in 30% glycerol. Staining results were visualized using a compound scope (Zeiss Axioscop, München, Germany) and digital camera (Zeiss AxioCam MRm, München, Germany). All sections used in this study consisted of heads, including mouthparts and antennae, and first and second thoracic segments including legs. We used positive staining of mouthparts (AL, DLL), legs (DLL) and antennae (DLL) to eliminate false negatives from our results.

RESULTS
Comparative morphology of horn development - prepupa to pupa
Horn primordia in both species grew explosively during the first 24 h of the prepupal stage, which in both species lasted approximately 48 h. At the onset of the prepupal stage, larval epidermis detached from the larval cuticle (Fig. 2). Horn primordia in both species formed through rapid cell proliferation of certain regions of the larval epidermis during the first 24 h of the prepupal stage. In both species cell proliferation occurred rapidly underneath the larval cuticle, which forced the growing tissue into folds. During the second half of the prepupal stage the prepupal epidermis began to excrete a visible cuticular layer that would form the pupal cuticle upon pupation. Once the animal molted into a pupa and shed the larval cuticle the prepupal epidermis and the newly excreted cuticle expanded to form the pupal body. Horn primordia expanded to form the pupal horns within approximately 2 h after pupation (Fig. 2). Apart from differences in location (Fig. 3), the dynamics of horn growth were thus similar in both species.

Comparative morphology of horn development - pupa to adult
The pupal stage lasted 9.02 ± 0.11 (n = 49) days in O. taurus and 10.93 ± 0.14 (n = 28) days in O. nigrientris at 25°C, respectively. In both species the pupal epidermis detached and retracted from the pupal cuticle within the first 4 days before depositing the final adult cuticle. Retraction of pupal epidermis was visible from the outside and did not require dissection. Magnitude of retraction differed markedly within and between species and not all horns or horn-like outgrowths present in pupae actually gave rise to horns in adults. In O. nigrientris, large male pupae expressed a long, downturned pronotal horn that gave rise to the adult horn without substantial modulation of horn size during the pupal stage (Fig. 3). Small male pupae expressed a smaller yet still substantial pronotal horn (Fig. 4A). In these males the pupal pronotal epidermis retracted somewhat, yet still gave rise to a prominent pronotal projection in the adult. All female pupae regardless of size also expressed a pronotal projection indis-
tistinguishable from that of small male pupae (Fig. 4A). Here, however, the pupal epidermis retracted during pupal development to a much greater degree than in small males prior to the deposition of adult cuticle, causing females to lack the adult pronotal outgrowths present in small males despite similar pupal morphologies (Fig. 4A).

Fig. 2. Prepupal growth of beetle horns. Left: schematic highlighting prepupal pronotal (thoracic) horn growth; right: corresponding 4',6-Diamidino-2-phenylindole stained sagittal sections of head capsule and first thoracic segment of a presumptive horned male Onthophagus nigriventris. (A) Prior to the prepupal stage the larval epidermis (blue) fully lines the larval cuticle (black). (B and C) At the onset of the prepupal stage larval epidermis detaches from the cuticle (indicated by arrows) and selected regions (indicated by asterisk) undergo rapid cell proliferation. The resulting tissue folds up underneath the larval cuticle. During the second half of the prepupal stage the epidermis secretes the future pupal cuticle. (D) Once the animal molts the folded epidermis and future pupal cuticle is free to expand and form the pupal thoracic horn.

Fig. 3. Species-specific proliferation of prepupal epidermis. Even congeneric species can differ markedly in the exact epidermal region that undergoes explosive growth during the prepupal stage. (A) Composite sagittal section through head and thorax of an Onthophagus taurus prepupa fated to develop into a major, horned male, and corresponding pupal morphology (inset). In O. taurus majors rapid growth of the head epidermis gives rise to a paired head horn (dashed arrow), while proliferation of the pronotal epidermis generates a single, central pronotal horn (solid arrow). Only the pupal head horns will subsequently give rise to adult structures while the pronotal horn will be reabsorbed entirely (see text and Fig. 4). (B) Composite sagittal section through head and thorax of an Onthophagus nigriventris prepupa fated to develop into a major, horned male, and corresponding pupal morphology (inset). In O. nigriventris majors the head epidermis (dashed arrow) does not undergo rapid growth, remains unfolded, and does not give rise to a horn in the prepupa, pupa or adult. In contrast, the pronotal epidermis (solid arrow) undergoes explosive growth and generates a single, central, down-curved pupal horn, which will give rise to a horn in the adult (see Fig. 5).
In *O. taurus*, only large male pupae expressed a pair of head horns, which gave rise to the paired head horns present in the adults without obvious modulation of horn size during the pupal stage (Fig. 4B). Smaller male *O. taurus* developed rudimentary pupal and adult head horns, whereas the heads of female pupae were always hornless. Variation in pupal head horn development therefore mirrored variation found among adults (Fig. 4B). However, all male and female *O. taurus* pupae regardless of size also expressed a substantial pronotal projection similar to that of *O. nigriventris* females and small males. Unlike *O. nigriventris*, however, neither female nor male *O. taurus* retained the pupal pronotal horn into adulthood. Instead, in all individuals observed the pupal pronotal epidermis retracted massively during the first half of the pupal stage prior to the deposition of adult cuticle, resulting in the development of a hornless adult pronotum in all cases (Fig. 4B).

Expression of *Dll* during prepupal horn development

In *O. nigriventris*, DLL protein was expressed in the distal portion of developing pronotal horn primordia in both male morphs (Fig. 5, A and B). Larvae fated to develop into the minor male morph expressed a smaller domain compared with their major, fully horned male counterparts (Fig. 5B). Interestingly, contrary to the distal expression domain found in males, female *O. nigriventris* expressed DLL in the proximal region of the prepupal pronotal horn (Fig. 5C). Since females, but not males, completely reabsorb their pronotal horn during the following pupal stage, this implicates the exact location of Dll expression as a possible regulatory mechanism. DLL protein was also detected in developing mouthparts and antennae, but not in the dorsal head epidermis of either males or females (not shown).

DLL protein was also detected in the growing head horns of *O. taurus*, which only develop in large male pupae and adults but never in females (Fig. 5D). In addition, we observed DLL protein in the distal-most half of the horn rudiment of incipient small, hornless males (Fig. 5E), but never in the corresponding region of the head epidermis of female *O. taurus* (Fig. 5F). Contrary to *O. nigriventris*, the prepupal pronotal horn primordium that in *O. taurus* grows in the prepupal stage but becomes reabsorbed during the pupal stage in all individuals, showed no signs of DLL expression regardless of sex and morph (Fig. 5F).

Expression of *al* during prepupal horn development

AL protein was absent from the developing head horns of incipient major *O. taurus* males, or the corresponding regions...
in minor males or females (data not shown). However, we detected AL protein in the pronotal horns of both male morphs and females in both species including the transitory horns of *O. taurus* (Fig. 6, A and B). We found no obvious differences in *al* expression patterns between morphs or sexes in both species. In each case, AL protein was found throughout the greater distal region of the pronotal horn primordium and clearly exceeded the domain of DLL expression described above (Fig. 6, A and B).

**DISCUSSION**

The origins of morphological novelties, and the interactions between genetic, developmental, and ecological mechanisms...
in their subsequent diversification, continue to represent two major frontiers in evolutionary biology (West-Eberhard 2003). Here we examined the development of beetle horns, a class of morphological traits that lack obvious homologs in other arthropods. Beetle horns are major, three-dimensional structures used as weapons in male combat over mating sites (Eberhard 1979; Emlen 1997). At the same time beetle horns exhibit remarkable diversity on a variety of levels such as alternative male morphs, sexual dimorphisms, or interspecific differences in the location of horns (Moczek 2005). This high level of morphological diversity within a narrow taxonomic frame provides an outstanding opportunity to characterize the mechanisms underlying novel morphological structures such as horns, as well as the changes to these mechanisms that have allowed these structures to diversify on different levels. Here we explored the regulation of horn development in two-horn polyphenic and sexually dimorphic species of horned beetles that differ in the body part that produces the adult horn. Several important observations emerged.

Differential retraction as a mechanism of horn size modulation

First, our results suggest that magnitude of horn expression is not only a function of horn growth during the prepupal stage as previously assumed (Emlen 2000), but also a function of the degree to which pupal horn primordia retract during the pupal stage prior to the deposition of the adult cuticle. The latter mechanism appears to be particularly important for pronotal (thoracic) horns, which can undergo differential retraction during pupal development depending on morph, sex, and species. For example, in O. nigriventris females and medium to small males express similar sized pupal horns. While in males these structures persist into the adult stage, sex-specific retraction during the pupal stage produces hornless females that lack horns completely. In O. taurus pupal thoracic horns are roughly similar in size to the head horns of large males in the same species, yet while the latter give rise to similar sized adult horns, the former are entirely re-absorbed during the pupal stage and leave no trace of their existence in the adult.

Conservation of patterning mechanisms in horn development

Our results also suggest that with the exception of the transitory pupal thoracic horns of O. taurus, horn primordia share at least one patterning gene, Dll, which, independent of location of the horn was expressed in the distal-most region of the developing prepupal horn. This suggests a conservation of function of DLL protein during beetle horn development compared with regular insect appendages, and is consistent with the hypothesis that proximo-distal axis formation in horns relies on the same or similar mechanisms as implemented in other arthropod appendages (Prpic et al. 2003). Dll expression in early appendage primordia or imaginal disks is regulated by the expression of two morphogens, wg and Dpp, whose expression in turn activates a set of at least in part conserved downstream targets (Panganiban et al. 1997; Nagy and Williams 2001). The timing and pattern of Dll expression therefore suggests that some upstream activators and downstream targets of Dll may also play a conserved role in the development of horns compared with regular appendages. Our data thus suggest that at least one, and maybe several, typical appendage patterning genes may also regulate the expression of beetle horns.

The origin(s) of sexual horn dimorphism

Our results raise the possibility that different species use different developmental mechanisms to generate dimorphic sexes. Female O. taurus never expressed Dll in the head epidermis that produces the horn in large males. Lack of Dll expression in the prepupal head epidermis might explain why female O. taurus never showed any indication of horn development as prepupae, pupae, and adults. In O. nigriventris, on the other hand, female prepupae did grow a pronotal horn and exhibited qualitatively similar expression patterns as their male counterparts, that is Dll and al were expressed in both sexes in the dorsal prepupal pronotal epidermis. Adult hornlessness in female O. nigriventris therefore cannot be attributed to the absence of DLL or AL protein in prepupal horn primordia. However, changes in the exact location of Dll expression may play a role in sexual horn dimorphism in O. nigriventris. While males expressed Dll protein in the distal-most region of the developing horn primordium, females showed the opposite pattern, that is, Dll was expressed in the proximal regions of the prepupal horn primordium. Since females, but not males, completely reabsorb their pronotal horn during the subsequent pupal stage, proximal Dll expression may still carry the same function as in males, that is, pattern which region of the prepupal pronotal epidermis will grow rise to the distal-most adult pronotal epidermis. In addition, this may designate prepupal epidermis anterior to this proximal Dll domain for retraction. If correct, evolutionary changes in the exact location of DLL expression (rather than presence/absence) would provide an important avenue for the modulation of horn size and degree of sexual dimorphism in at least some species.

The origin(s) of male horn polyphenism

Both male morphs in both O. taurus and O. nigriventris expressed Dll in the distal-most region of the developing horn or horn rudiment, respectively. Differences in horn development among major and minor morphs in both species therefore do not appear to be due to the presence or absence of DLL protein in the head or pronotal epidermis. Here, differences in
horn development might be unrelated to *Dll* expression and because of other horn patterning genes that remain to be examined. Alternatively, rather than presence or absence of expression, differences in the exact timing of expression of *Dll* or other patterning genes might also be important. Horn primordia grow explosively during a very narrow time window, and even minor differences in the exact length of growth periods may be sufficient to generate major morphological differences (Nijhout and Wheeler 1996).

**Interspecific diversity in the location of horn expression**

Lastly, our results indicate that different types of horns may be patterned by a different set of patterning genes. Prepupal head horns in *O. taurus* never expressed AL protein, whereas prepupal thoracic horns in both species expressed AL protein. Interestingly, in those horns in which AL protein was detected the domain of AL expression exceeded the corresponding DLL domain in each case, which is contrary to most other arthropod appendages known to be patterned by both transcription factors or where expression patterns are available (e.g., Campbell and Tomlinson 1998; Kojima 2004, but see Miyawaki et al. 2002). Work is underway to move towards a functional analysis of beetle horn patterning mechanisms, which will help determine the significance, if any, of these observations.

**Conclusions**

Combined, our results begin to paint a complex picture of the developmental regulation and evolutionary origin of beetle horns and horned beetle diversity. Our results suggest that beetle horns share some of the same patterning genes with traditional insect appendages (Campbell et al. 1993; Pangani-ban and Rubenstein 2002). At the same time, our results suggest that even among closely related species, and even within the same species and individual, different types of horns may have different developmental origins, and may have had different evolutionary histories. The presence of pronomal horns in prepupae and pupae of female and minor male *O. nigriventris*, and their subsequent differential retraction during the pupal stage suggest a fundamentally different mechanism in the development of alternate male morphs and sexual dimorphisms compared with the development of head horns in *O. taurus*, where head horn expression appears to be exclusively a function of sex specific horn induction during the pupal stage. These observations also raise the possibility that ancestrally, adult pronomal horns may have been present regardless of adult body size in both sexes of both species. Subsequent genetic changes in the degree of prepupal horn growth and pupal retraction of horn primordia then shaped intra- and intersexual dimorphisms in *O. nigriventris*, while in *O. taurus* adult pronomal horns appear to have been lost altogether via complete retraction of pupal horn primordia during the pupal stage. Head horns in *O. taurus*, in contrast, appear to have originated as a sex specific trait. Male head horns originally may have been expressed in all males regardless of size, and subsequent genetic changes repressing horn expression in small males, and permitting or elevating it in large males may have given rise to the pronounced male polyphenism we observe today. The notion that different types of horns in beetles evolved independently in the same clade is also beginning to receive support from phylogenetic analyses of horned beetles (Moczek 2005).

More generally, our results are in line with a large number of studies that suggest that the origin of morphological novelties rests at least in part on the redeployment of already existing developmental mechanisms, such as appendage patterning processes in the case of horns in beetles. Our results also suggest, however, that little to no phylogenetic distance is needed for the evolution of very different modifier mechanisms that allow for substantial modulation of trait expression at different time points during development in different species, sexes or tissue regions of the same individual. These findings suggest that developmental evolution on the level of populations and closely related species can be remarkably diverse and can generate substantial phenotypic diversity, possibly sufficient to fuel large-scale macroevolutionary transitions over time.

**Acknowledgments**

We would like to thank D. Rose for helpful comments on earlier versions of this manuscript, C. Jacobson for collecting *O. nigriventris*, and G. Burd for generously providing access to a cryostat. We also would like to thank Maple View Farm, North Carolina, and the University of Arizona Agricultural Station for their continued support in providing beetles and beetle food. This study was funded in part through a National Institutes of Health Postdoctoral Excellence in Research and Teaching Fellowship (NIH Training Grant # 1K12GM00708) and NSF Grant IOB 0445661 to APM, and NSF Grant IBN 9874624 to LN.

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