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ORIGINAL ARTICLE

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Conservation, innovation, and the evolution of horned beetle diversity

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Abstract Beetle horns represent an evolutionary novelty exhibiting remarkable diversity above and below the species level. Here, we show that four typical appendage patterning genes, *extradenticle (exd)*, *homothorax (hth)*, *dachshund (dac)*, and *Distal-less (Dll)* are expressed in the context of the development of sexually dimorphic thoracic horns in three *Onthophagus* species. At least two of these genes, *Dll* and *hth*, exhibited expression patterns consistent with a conservation of patterning function during horn development relative to their known roles in the development of insect legs. *exd*, *hth*, and *dac* expression patterns during horn development were largely invariable across species or sexes within species. In contrast, *Dll* expression was far more discrete and exhibited consistent differences between sexes and species. Most importantly, differences in location and domain size of *Dll* expression tightly correlated with the degree to which prepupal horn primordia were retained or resorbed before the final adult molt. Our results lend further support to the hypothesis that the origin of beetle horns relied, at least in part, on the redeployment of already existing developmental mechanisms, such as appendage patterning processes and that changes in the exact location and domain size of *Dll* expression may represent important modifier mechanisms that modulate horn expression in different species or sexes. If correct, this would imply that certain components of genetic basis of horn development may be able to diversify rapidly within lineages and largely independent of phylogenetic distance. We present a first model that integrates presently available data on the genetic regulation of horn development and diversity.

Keywords Appendage patterning · *Distal-less* · Evolutionary novelty · Lability · Sexual dimorphism

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Introduction

A major challenge in evolutionary developmental biology is to understand if and how developmental evolution on the level of populations and closely related species mediates macroevolutionary transitions and the origin of evolutionary novelty. Microevolutionary approaches to development, such as artificial selection experiments, common garden breeding, or hybridization experiments, all indicate that many developmental processes evolve readily on the level of populations and closely related species (Zera and Zhang 1995; Beldade and Brakefield 2002; Zijlstra et al. 2004). While such short-term developmental evolution clearly contributes to natural phenotypic diversity, it has been difficult to link the resulting subtle and quantitative changes in phenotype expression to the emergence of major novel phenotypes that characterize differences between higher-order taxa. Instead, the origin of novel phenotypes has, thus far, most commonly been approached through comparative studies on the level of phyla or classes. Such studies have generally come to emphasize the role of redeployment and subsequent modification of already existing developmental processes in a novel context as an important source of evolutionary innovation. This macroevolutionary approach to development has been crucial for explaining how a limited set of developmental processes can become modified to ultimately generate a wide range of phenotypic diversity (reviewed in True and Carroll 2002). At the same time, however, this perspective has provided little insight into the mechanisms that mediate such redeployment or the phylogenetic distances necessary for the evolution of modifier mechanisms capable of

adjusting phenotype expression in a novel developmental context. Consequently, exactly how micro- and macroevolution of development relate to one another remains largely unclear, and we have yet to learn to what degree microevolutionary changes in developmental processes ultimately suffice for the origin of major evolutionary novelties (Raff 1996, Schlichting and Pigliucci 1998; Gilbert 2001; West-Eberhard 2003; Davidson and Erwin 2006). Here, we address these issues by exploring the developmental genetic basis of a class of traits that is both novel and highly diverse: beetle horns.

Beetle horns are one of the most diverse classes of secondary sexual traits. Thousands of species belonging to at least six families of beetles express horns or horn-like structures, with the most extreme and diverse expression being found in the chafers, or scarab beetle family (Arrow 1951). Thus far without exception, and independent of their exact shape or location, beetles use their horns as weapons in male combat over access to females, and their functional significance and fitness consequences have been well demonstrated (reviewed in Emlen 2000; Moczek 2005, 2006a,b). At the same time, beetle horns lack obvious homology to other structures in and outside the insects (Moczek and Nagy 2005). Beetle horns can, therefore, be considered an example of an evolutionary novelty that provides its bearer with a significant new phenotype: a powerful weapon in male–male competition.

Beetle horns are extraordinarily diverse. Horns range from small knobs or protrusions on a beetles' head or thorax to gigantic outgrowths that account for up to 30% of a beetle body mass and completely transform the shape of their bearer (Moczek 2005). Interestingly, such dramatic diversity in size and location of horn expression is not confined to the comparison of distant families or genera, but commonly exists among closely related species in the same genus or even within individual species (Emlen et al. 2005a,b). For example, in almost all species of horned beetles, horn expression is confined to males and is greatly reduced or absent in females. This remarkable phenotypic diversity within a very narrow taxonomic framework opens up the possibility to investigate not only the genetic, developmental, and ecological mechanisms that enabled the origin of horns, but also how modifications of these mechanisms and the interactions between them, have mediated the diversification of horns below and above the species level. Here, we explore the developmental genetic basis of horn development in three sexually dimorphic *Onthophagus* species.

Beetle horns originate from selected regions of the larval epidermis during the prepupal stage late in larval development (Emlen and Nijhout 1999; Moczek and Nagy 2005). At the onset of the prepupal stage, the larval epidermis detaches from the larval cuticle and selected epidermal regions undergo rapid cell proliferation, which forces the resulting tissue into folds. During the second half of the prepupal stage, the prepupal epidermis begins to excrete a visible cuticular layer that will eventually sclerotize into the pupal cuticle upon pupation. Once the animal molts into a pupa and sheds the larval cuticle, the prepupal epidermis and newly excreted cuticle then expand to form the pupal body (Moczek 2005, 2006a). Although beetle horns lack muscles, joints, and nervous tissue, their epidermal origin and rapid prepupal growth are reminiscent of the development of more traditional insect appendages such as legs, mouthparts, or antennae. This observation suggested that horn development may be patterned by some of the same patterning elements that instruct the formation of other insect appendages (Moczek and Nagy 2005).

A large number of studies have shown that the remarkable diversity of arthropod appendages is, at least in part, patterned by a remarkably conserved gene network (Panganiban et al. 1994, 1997; Nagy and Williams 2001; Kojima 2004). This network has been, by far, best studied in *Drosophila*, where appendages develop from imaginal discs; monolayered groups of epidermal cells set aside from larval tissues during embryogenesis. During the late second to early third larval instar, two diffusible morphogens, *wingless (wg)* and *decapentaplegic (dpp)* interact in a concentration-dependent manner, resulting in the subdivision of imaginal disks into roughly concentric and nested domains of expression of the transcription factors, *Distal-less (Dll)*, *dachshund (dac),extradenticle (exd)* 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 nuclear *hth*/*exd* expression, pattern progressively more proximal appendage regions (Abu-Shaar and Mann 1998; Lecuit and Cohen 1997; Wu and Cohen 1999; Kojima 2004). In many other arthropods, adult appendages develop not from imaginal disks but via the outbudding of selected epidermal regions during larval development, and thus, are more similar to the way beetle horns develop (e.g., Fristrom and Fristrom 1993; Nagy and Williams 2001; Moczek and Nagy 2005). Despite these differences in appendage development, the molecular mechanisms used to pattern appendages derived from epidermal outbuddings are similar. For example, *Dll* activity is functionally required for distal leg formation in beetles and spiders (Beermann et al. 2001; Schoppmeier and Damen 2001), and *Dll* expression in the distal region and *hth* expression in the proximal region occur during the development of appendages in a wide range of insects and noninsect arthropods (reviewed in Angelini and Kaufman 2005). Here, we examine the expression of *Dll*, *dac*, *exd*, and *hth* in the context of thoracic horn development and the development of sexual dimorphism in three closely related species in the horned beetle genus, *Onthophagus*. In particular, we address three major questions: First, are *Dll*, *dac*, *exd*, and *hth* expressed in the context of thoracic horn development? Second, is the pattern of expression of these patterning elements consistent with a conservation of function compared to more traditional appendages? Third, which, if any, changes in the expression of these patterning elements are associated with differences in horn development between sexes and species?

Materials and methods

Species choice

We investigated thoracic horn development in three congeneric *Onthophagus* species. All three species differ in horn morphology and nature and extent of sexual dimorphism. In *Onthophagus nigriventris* (Figs. 1, 2), large adult males develop a very long, thin, and curved thoracic horn, whereas, females develop a broad, minor ridge. In *Onthophagus binodis*, large males develop a broad, relatively short, and straight pronotal (thoracic) horn, whereas, females again develop only a broad, minor ridge. Males and females of the third species, *Onthophagus taurus*, never express thoracic horns as adults, and horn development is instead restricted to the heads of large males (examined in Moczek and Nagy 2005). However, earlier observations suggested that *O. taurus* may actually grow a thoracic horn primordium during earlier developmental stages but may fail to retain it into the adult stage (Figs. 1, 2; see also Moczek 2005; Moczek and Nagy 2005).

Beetle rearing

Beetle laboratory colonies were derived from animals collected from pastures near Waimea, Hawaii (*O. nigriventris*, *O. binodis*), and Durham, North Carolina (*O. taurus*). All laboratory colonies were kept in growth chambers at Indiana University at 23°C under a 16:8 light:dark cycle. Colony maintenance, breeding, and sexing of larval and adult beetles were as described previously (Moczek and Nagy 2005). 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 (see below). Prepupae were fixed and cryosectioned as described in Moczek and Nagy (2005).

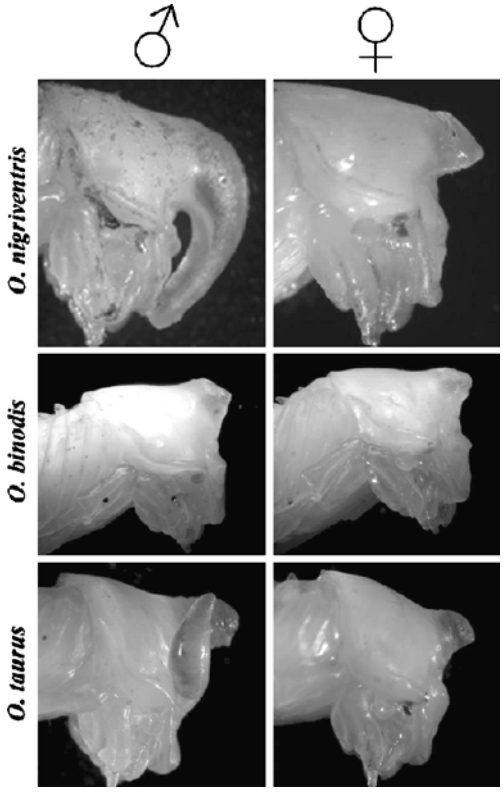


Fig. 1 Pupal head and thorax morphologies of male (*left*) and female (*right*) *Onthophagus nigriventris* (*top*), *O. binodis* (*center*), and *O. taurus* (*bottom*). Note that both sexes in all three species express thoracic horns during the pupal stage

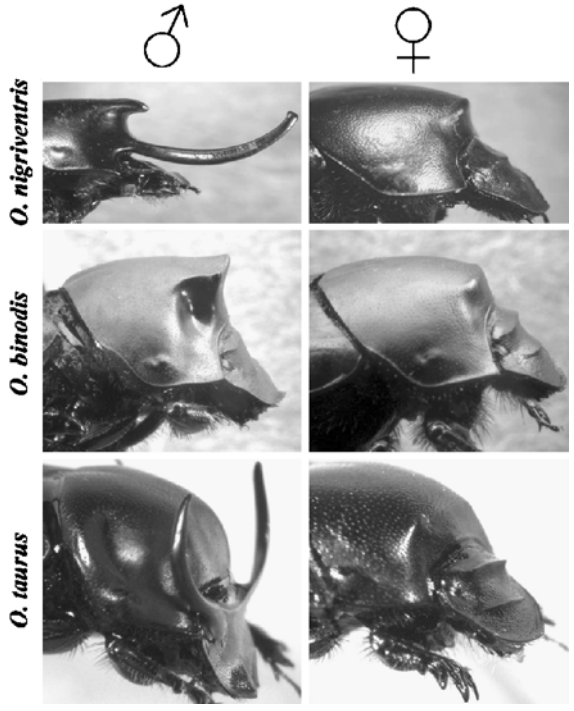


Fig. 2 Adult head and thorax morphologies of male (*left*) and female (*right*) *Onthophagus nigriventris* (*top*), *O. binodis*(*center*), and *O. taurus* (*bottom*). Note that only male *O. nigriventris* and *O. binodis* express fully developed thoracic horns as adults, whereas, their female counterparts express only rudimentary thoracic ridges. Furthermore, note that both sexes in *O. taurus* show no indication of thoracic horn growth in the adult despite the expression of a conspicuous thoracic horn in the preceding pupal stage (Fig. 1)

Immunohistochemistry

We investigated the distribution of DLL, HTH, and EXD proteins by immunohistochemistry using antibodies previously shown to recognize these proteins in diverse species (DLL: Panganiban et al. 1994; HTH: Kurant et al. 1998; EXD: Aspland and White 1997). Microscope slides with sections were equilibrated to room temperature (RT). Sections were washed 3× with phosphate buffered saline +0.1% Triton X-100 (PBT) for 10 min per wash. Sections were then incubated with 2% (or 4% for EXD) bovine serum albumin in PBT for 4–5 h at RT, followed by two

quick washes with PBT. Sections were then incubated in primary antibody (EXD 1:5; DLL 1:100; HTH 1:500) overnight at 4°C. Sections were then washed at RT 3× quickly with PBT, followed by two washes for 25 min each. Sections were then incubated in anti-mouse (EXD), anti-rabbit (DLL, HTH) secondary CY3-conjugated antibody, diluted 1:200 in blocking solution for 2 h. Sections were then washed 3× fast in PBT followed by 2 washes in 15 minute intervals. Sections were counterstained with DAPI at 1:1,000 in PBT (Sigma-Aldrich; St. Louis, MO) for 15 min, washed fast 3× and 1× for 15 min with PBT and mounted in 30% glycerol (Aqua PolyMount Polysciences; Warrington, PA). Stainings were visualized on an Eclipse E800 epifluorescence microscope (Nikon Tokyo, Japan) and images were collected with a Hamamatsu ORCA-ER digital camera with MetaMorph imaging software (Molecular Devices Sunnyvale, CA) and processed using Photoshop 7.0 (Adobe Systems, Palo Alto, CA). All sections used in this study consisted of heads, including mouthparts and antennae, and first and second thoracic segments including legs. Evidence of previously published staining patterns in legs, mouthparts, and legs was used as a positive control.

Dachshund cloning

Total RNA was extracted from head and thorax tissue of *Onthophagus taurus* prepupae by homogenization with a Polytron PT3000 (Brinkmann) in 1.0-ml TRIzol (Invitrogen). cDNA was created using Clontech's MatchMaker 2 kit and nested degenerate primers designed by Prpic et al. (2001) were used to clone *Otdac*. Standard nested degenerate PCR was performed and the products were cloned into pCR-II-TOPO (Invitrogen). Products were sequenced three times by automated sequencing (GATC DNA Sequencing Facility, University of Arizona) in both directions. We isolated a region of the *Otdac* gene (accession number DQ452570) containing an ORF of 966 bp (322 amino acids). We designed gene specific primers based on this sequence and were able to recover the same fragment from newly synthesized *Otc*DNA.

Sequence analysis

Sequence analysis and orthology assignment were performed using the National Center for Biotechnology Information (NCBI) BLAST [blastx] program and GCG/Seqlab (Wisconsin Package). The *Ot* DAC putative protein coding sequence was compared to the DAC putative protein coding sequences from *Drosophila* and *Tribolium* using the LALIGN (Pearson 1991) sequence comparison program. Dac sequences were aligned using ClustalW (Thompson et al. 1994) and the BLOSUM 62 residue comparison matrix (Henikoff and Henikoff 1992) assigning a gap-opening penalty of 10 and a gap extension penalty of 0.2. Dendrogram was generated using parsimony analysis utilizing a heuristic search, TBR with 2,000 random additions, and nonparametric bootstrap with 1,000 replicates (200 random additions/replicate) as implemented in PAUP 4.0 b10 (Swofford 2003).

Riboprobe synthesis and in situ hybridization

We used the same riboprobe derived from a 966-bp fragment of *O. taurus dachshund* to visualize *dac* expression in *O. taurus*, *O. nigriventris*, and *O. binodis*. Preliminary in situ hybridizations showed a very similar pattern of *dac* expression in medial leg regions of all three species (not shown). This observation suggested that *dac* expression is conserved in *Onthophagus* legs and that our probe reliably detects *dac* expression across closely related species. In situ hybridization protocols largely followed Tautz and Pfeifle (1989) and Klingler and Gergen (1993). DIG-labeled (Roche, Indianapolis IN) sense and antisense riboprobes were synthesized with T7 and SP6 RNA polymerases using PCR amplified *Otdac* and then hydrolyzed to a final length of approximately 250 bases using a 2× carbonate buffer. Slides containing *Onthophagus* sections were incubated with hybridization buffer containing equal concentrations of either sense (T7) or antisense (Sp6) denatured riboprobe overnight at 58°C. Sections were then washed at 58°C for 25 min, blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature, incubated with anti-Dig antibody solution (Roche) for 1 h at room temperature, and washed with PBT 6× for 10 min each. To visualize signal, hybridized slides were incubated with an alkaline phosphatase-conjugated antibody to the DIG label, and subsequently, stained in an alkaline phosphatase buffer containing the color-producing substrates, nitroblue tetrazoleum (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Pierce). Color reactions were stopped with PBTween (PBS with 0.1% Tween 20 (Calbochem) and then nuclear-stained with 1:1,000 solution of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) in PBTween for 20 min. Slides were sealed with Aquapolymount (Poly-Sciences) and stored at −20°C. Stained sections were viewed using fluorescent (DAPI) and brightfield microscopy (Axioplan, Zeiss, Jena, Germany) and digital images were captured with a SPOT RT digital camera using SPOT software (Diagnostic Instruments, Sterling Heights MI, USA).

Results

Horn development during the prepupal stage

Males and females of all three species grew a moderate to large thoracic horn during the prepupal stage regardless of final adult morphology (Fig. 1). The thoracic horn was clearly visible in all pupae. Large *O. nigriventris* males grew the largest thoracic horn primordia, followed by male *O. taurus* and male *O. binodis*. Females also grew thoracic horn primordia in all three species. Thoracic horn primordia in female *O. nigriventris* and *O. taurus* were

considerably smaller compared to those of their male counterparts, whereas, female *O. binodis* grew prepupal thoracic horns similar in size compared to horn primordia observed in male *O. binodis* (Fig. 1).

Horn development during the pupal stage

Species differed substantially in the degree to which they retained horn primordia grown during the prepupal stage into the final adult stage. *O. nigriventris* and *O. binodis* males retained their pupal thoracic horn and molted into horned adults without exception. Females of both species, instead, resorbed most of their horn during the pupal stage and molted into largely thorax-hornless adults. Pupal horn resorption was most extreme in *O. taurus* and gave rise to adults lacking any signs of the former presence of a thoracic outgrowth. In addition, and in contrast to the other two species, pupal horn resorption in *O. taurus* occurred in both sexes and resulted in the secondary loss of the thoracic horns in females and in males (Fig. 2; for a quantitative examination of pupal remodeling see Moczek, in review).

Dachshund cloning and sequence analysis

We isolated a region of the *Otdac* gene by degenerate PCR, which yielded a clone containing an ORF of 966 bp (322 amino acids). Amino acids 1–247 of *Ot* DAC had 53.6% amino acid identity to *Drosophila* DAC (amino acids 278–557) and amino acids 278–322 of *Ot* DAC had 45.7% amino acid identity to *Drosophila* DAC (amino acids 715–760) (Fig. 3a). The relatively close relationship between two beetles, *O.taurus* and *Tribolium castaneum*, was reflected in the degree of similarity of their nucleotide sequences (65.2%) as well as their putative DAC protein coding sequences, with *Ot* DAC (amino acids 1–322) sharing 78.7% amino acid identity with *Tribolium* DAC (amino acids 1–301). The cloned portion of *Ot* DAC included portions of the two conserved domains (Dachshund Domain 1 and 2, DD1 and DD2) found in all DAC protein sequences for which this region has been cloned. The first 44 amino acids of *Ot* DAC constitute the C-terminal region of the DD1, and the last 14 amino acids of *Ot* DAC contain the N-terminal portion of the DD2. Furthermore, two putative Nuclear Localization Signals are present in the *Drosophila* sequence and of those only one (indicated in Fig. 3a as NLS) is key to Dachshund nuclear transport and function (Tavsanli et al. 2004). A sequence of 16 amino acids, WENCRAAYEDIVKHLE (indicated in Fig. 3a as insect motif), is only present in DAC from insect species and is absolutely conserved. This motif has not been previously reported and its functional significance, if any, is unknown.

Expression of *dac* during prepupal horn development

We investigated *dac* expression across sexes and species via in situ hybridization using a hydrolyzed 966-bp riboprobe to the *O. taurus dac* ortholog. *dac* expression was detected in the posterior-medial region of developing legs of all three species, consistent with a conservation of *dac* function in the patterning of medial appendage identity during *Onthophagus* leg development (not shown). However, similar to the expression of EXD (see below), we were unable to detect any clear subdivision with respect to *dac* expression during prepupal thoracic horn growth. Instead, *dac* expression occurred throughout the thoracic horn primordium regardless of species, sex, or final adult horn morphology (Fig. 4). Furthermore, *dac* expression extended throughout most of the dorsal and ventral epidermis proximal to antennae and mouthparts in the head and legs and wings in the thorax (not shown).

Expression of HTH during prepupal horn development

We investigated HTH expression through immunohistochemistry using a pangenic antibody (Kurant et al. 1998). The expression of HTH protein was similar in prepupal thoracic horns of all three species regardless of sex and whether or not horns persisted into adulthood (Fig. 5). In each instance, HTH protein exhibited a strong proximal domain of expression and was detected at high levels at the anterior and posterior base of the prepupal horn (Fig. 5). In a small fraction of sections obtained from *O. taurus* and *O binodis*, and the majority of sections obtained from *O. nigriventris*, low levels of HTH expression were also detected in the remainder of thoracic horn primordium including the future distal region (shown for *O. nigriventris* in Fig. 5). Additionally, in the majority of sections obtained from all three species HTH protein was also detected in the proximal, but not distal, regions of developing mouthparts, antennae and legs (not shown).

Expression of nuclear *exd* during prepupal horn development

We investigated nuclear EXD (n-EXD) expression through immunohistochemistry using a pangenic antibody (Aspland and White 1997). n-EXD was detected in the proximal (but not distal) region of developing *Onthophagus* legs, consistent with a conservation of *exd* function in the patterning of proximal appendage identity during *Onthophagus* leg development (not shown). However, no clear and consistent subdivision of n-EXD expression was observed during pronotal horn development. In *O. binodis* and *O. nigriventris*, n-EXD expression typically occurred throughout the thoracic horn primordium regardless of sex or final adult horn morphology (Fig. 3). Only *O. taurus* sections exhibited an n-EXD pattern of expression similar to that of HTH expression, i.e, the majority of sections

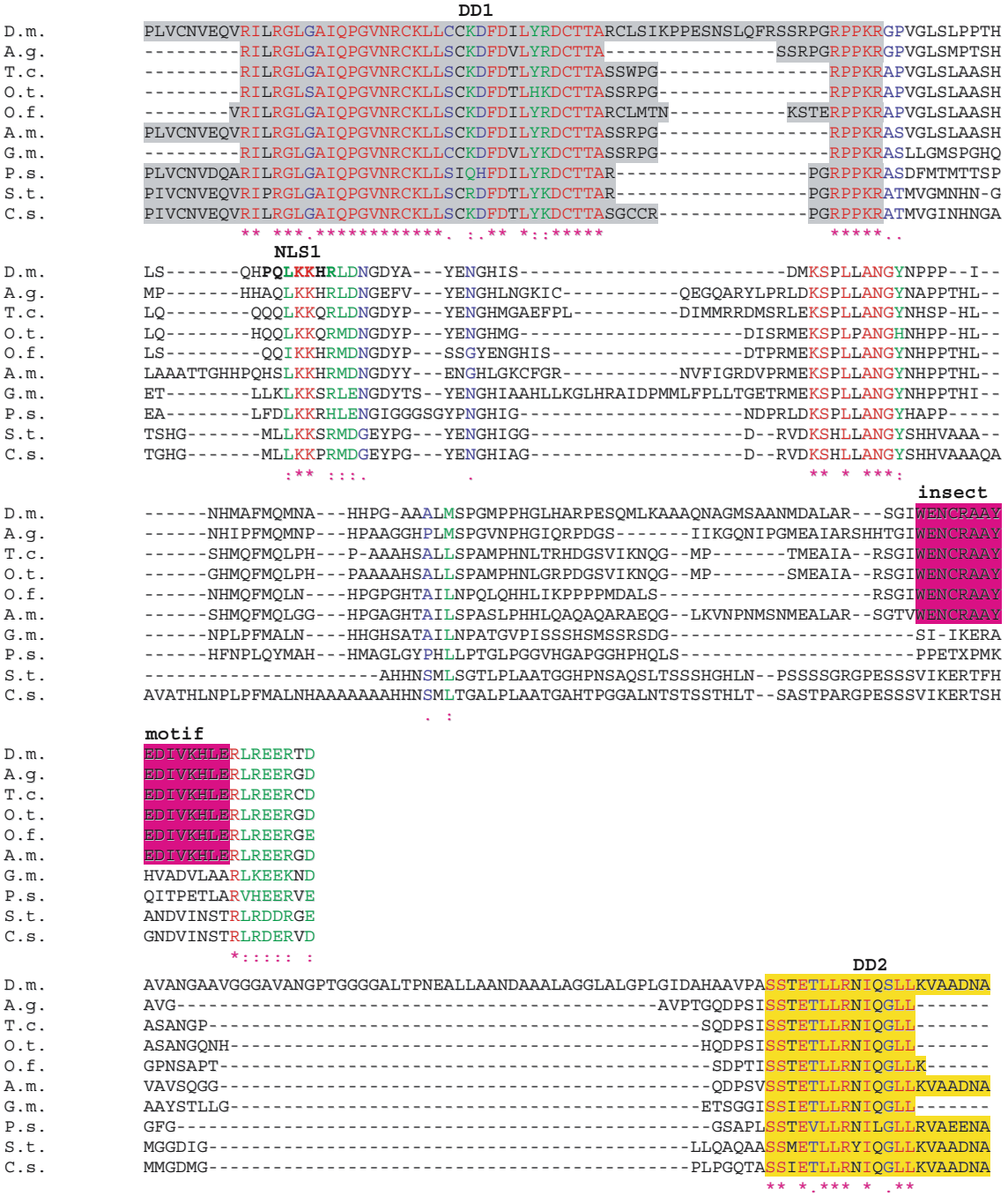
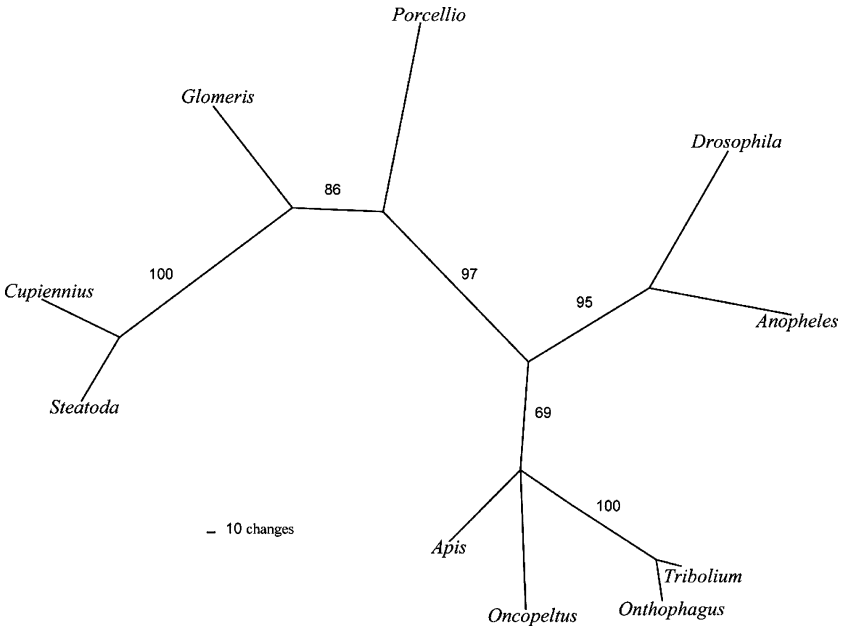


Fig. 3 **a** Alignment of arthropod *Dachshund* sequences. Sequences shown are those lying within portions of the ascribed DD1 and DD2 domains (Davis et al. 1999). Regions of identity are represented by *vertical dashes*, conserved substitutions are represented by a *vertical double dot*, and semi-conserved substitutions represented by a *single dot*. Alignment was generated using ClustalW (Pôle BioInformatique Lyonnais). Abbreviations for animals and accession numbers: *D.m. Drosophila melanogaster*-AAC46506.1, *A.g. Anopheles gambiae*-XP_317545, *T.c. Tribolium castaneum*-CAC84070.1, *A.m. Apis mellifera*-XP_394482.2, *O.f. Oncopeltus fasciatus*-AAS93632.1, *P.s. Porcellio scaber*-AAK58707.1, *S.t.Steatoda triangulosa*-AAK58706.1, *C.s. Cupiennius salei*-CAD57736.1, *G. m. Glomeris marginata*-CAD82906.1. Region *highlighted in gray*=C-terminal region of the DD1 domain; NLS1 (amino acids *bold/shadowed*)=Nuclear localization signal 1; region *highlighted in pink*=insect motif; region *highlighted in yellow*=N-terminal portion of the DD2 domain. See text for further information. **b** Phylogram of arthropod *Dachshund* protein sequences. Only sequences lying within portions of the ascribed DD1 and DD2 domains (Davis et al. 1999) were used for the phylogenetic analysis. A consensus tree of the three most parsimonious topologies (branch lengths from a single optimal topology) was used to generate a dendrogram, which is an unrooted majority rule consensus computed using parsimony analysis. Numbers along branches represent bootstrap values

Fig. 3 (continued)



exhibited stronger expression in the anterior and posterior base of the horn, and reduced expression in the distal horn region. Weak to moderate EXD expression was also observed in all three species throughout large portions of the dorsal pronotal epidermis adjacent to the region of horn growth (not shown).

Expression of *Dll* during prepupal horn development

In contrast to *hth*, *dac*, and *exd*, prepupal *Dll* expression exhibited marked differences depending on species, sex, and whether or not horns persisted into adulthood. In male *O. nigriventris*, which retained their entire prepupal and pupal horn into adulthood, DLL protein was expressed in the distal portion of developing pronotal horn primordia (Fig. 6). In contrast, female *O. nigriventris*, which resorb a large fraction of their thoracic horn during the pupal stage, expressed DLL protein in a far more *posterior* region of the prepupal pronotal horn (Fig. 6). Lastly, in *O. taurus*, in which pupal horn resorption is most extreme and occurs in both sexes rather than just one, DLL expression was limited to a small domain near the posterior base of the horn primordium, but absent from the remainder of the outgrowth. In *O. taurus*, this pattern was observed in both female and male prepupae (Fig. 5) and contradicts earlier results on pronotal DLL expression in this species (Moczek and Nagy 2005).

Discussion

The developmental and genetic mechanisms that mediate the origins of morphological novelties are of major interest to evolutionary biologists (West-Eberhard 2003). We examined the development of beetle horns, a class of traits lacking obvious homology to other arthropod structures. At the same time, beetle horns exhibit remarkable diversity on a variety of levels such as sexual dimorphisms or interspecific differences in shape, size, or location of horn growth (Moczek 2005). This high level of morphological diversity within a narrow taxonomic frame provides an excellent opportunity to explore the mechanisms underlying novel morphological structures such as horns and the changes in these mechanisms that have allowed these structures to diversify on different levels. Here, we explored the regulation of horn development in three closely related and sexually dimorphic species of horned beetles. Several important observations emerged.

Secondary loss of horn primordia is an important mechanism of horn size modulation

Our results confirm and expand earlier observations (Moczek and Nagy 2005) that differential loss of horn primordia during the pupal stage, and thus, well after their original prepupal growth phase constitutes a widespread mechanism underlying the development of sexual dimorphism in horned beetles. In *O. nigriventris*, resorption of female but not male horn primordial tissue further exaggerated sexual dimorphism in horn expression already generated by differential prepupal growth (for a quantitative analysis see Moczek, in review). In contrast, resorption of female but not male pupal horn primordial tissue was the main, if not sole, mechanism generating sexual horn dimorphism in *O. binodis*, in which both sexes initially had grown very similarly sized horns. In *O. taurus*, pupal horn resorption had an altogether different effect and *eliminated* initial sexual dimorphism in thoracic horn expression (visible between male and female pupae) via the wholesale resorption of thoracic horn primordia in both females and males.

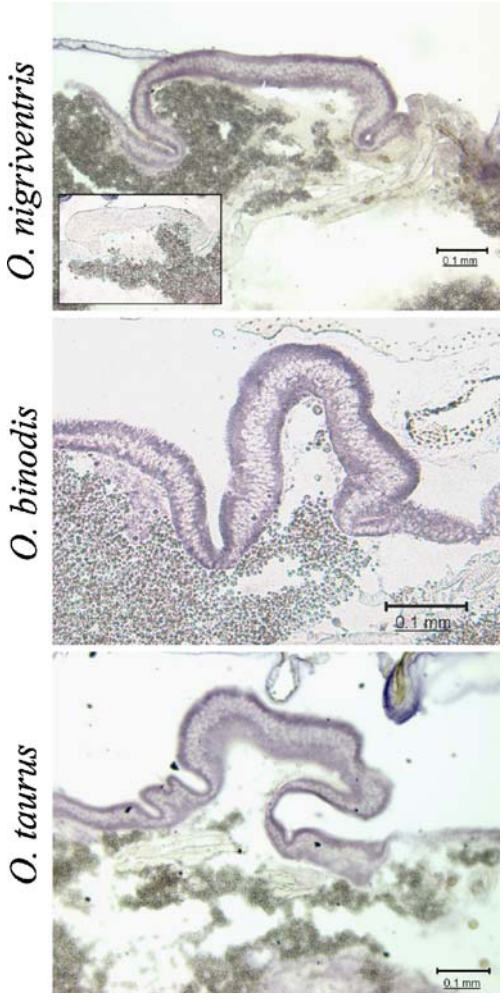


Fig. 4 Expression of *dac* in prepupal horn primordia of *O. nigriventris* (*top*), *O. binodis* (*center*), and *O. taurus* (*bottom*). *dac* is expressed throughout the thoracic horn primordium irrespective of species and sex, and only one sex per species is shown. All individuals were approximately 24-h-old prepupae. *Dorsal* is *up*, and *anterior* is to the *right*. *Inset*: Representative sense-control result. Corresponding sense control images are available for all species and sexes upon request

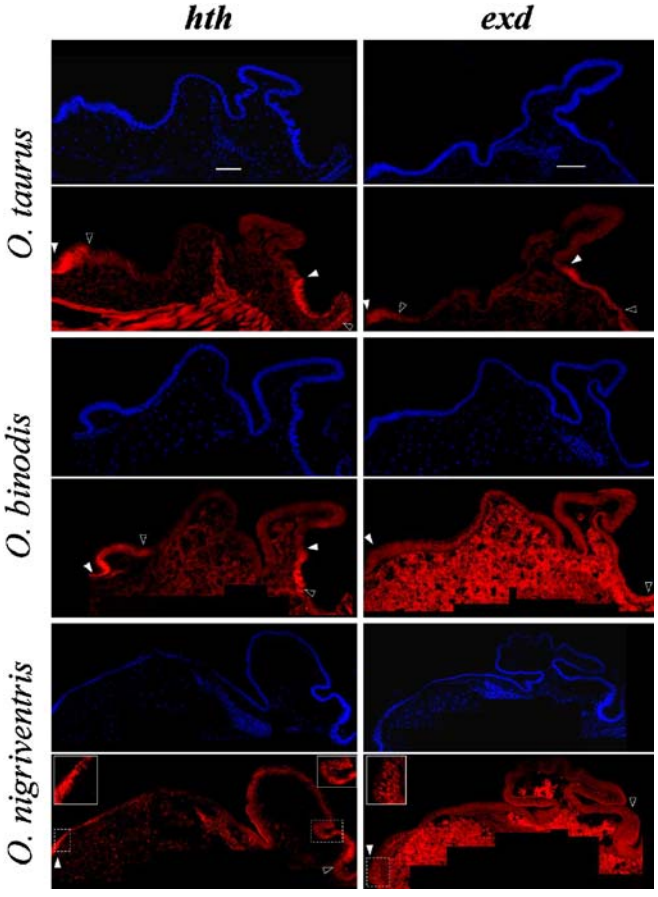


Fig. 5 Expression of HTH and EXD in prepupal horn primordia in *O. taurus* (*top*), *O. binodis* (*center*), and *O. nigriventris* (*bottom*; *blue*: nuclear counter stain, DAPI; *red*: HTH/nuclearEXD expression; *dorsal* is *up*, and *anterior* is to the *right*.). Expression patterns did not differ between sexes and only one sex per species is shown. *Left*: In all three species HTH was expressed strongly in anterior and posterior proximal regions of the future horn. *Right*: n-EXD did not exhibit obvious and consistent differential expression during horn development with the possible exception of *O. taurus* (*top*), wherein the majority of animals exhibited much stronger EXD expression in proximal regions similar to those that express HTH. In the other two species, n-EXD expression was largely ubiquitous and occurred throughout the horn primordium and the pronotal epithelium adjacent to the site of horn growth. *Solid and open arrowheads* indicate the *posterior*- and *anterior*-most limit of the expression domain of interest as it is visible in the figure. *Inserts* highlight areas of gene expression in *O. nigriventris* where interference through non-specific signal was particularly severe. *p→d* indicates the approximate orientation of the proximo-dostal axis along which the horn primordium will unfold during pupal ecdysis

Our results also suggest that this mechanism is evolutionarily labile and that even closely related species can differ dramatically in the degree to which they employ this mechanism in one or both sexes to modulate the expression of secondary sexual traits. Pupal horn resorption is achieved via programmed cell death (Kijimoto, Andrews, Moczek, in preparation) and its differential activation and regulation in different sexes and species is currently being investigated. Apoptotic loss of appendage primordia has previously been documented in *Pheidole* ants in which apoptosis mediates the caste-specific degeneration of wing discs during the prepupal–pupal transition (Sameshima et al. 2004) and in some Lepidoptera in which winglessness in adult females is achieved developmentally via sex-specific apoptotic wing degeneration during pupal development (Niitsu 2001).

Patterning mechanisms of beetle horn development: conservation with modification

Our results also show that four typical appendage patterning genes, *exd*, *hth*, *dac*, and *Dll* are expressed in the context of the prepupal development of sexually dimorphic thoracic horns in *Onthophagus* beetles. HTH expression was strongest in future proximal horn regions, and thus, similar to the typical proximal domain of expression documented in a wide range of appendage types in insects and noninsect arthropods (Angelini and

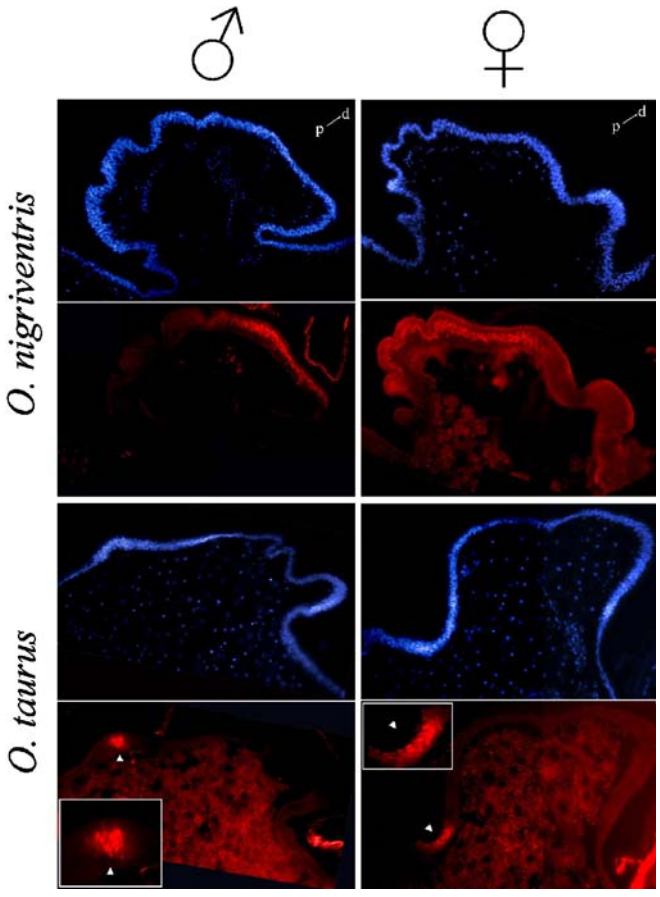


Fig. 6 Expression of DLL in prepupal horn primordia in male (*left*) and female (*right*) *O. nigriventris* (*top*) and *O. taurus* (*bottom*; *blue*: nuclear counter stain, DAPI; *red*: nuclear DLL expression; *dorsal* is *up*, and *anterior* is to the *right*.). *Top*: In male *O. nigriventris* (*left*) DLL is expressed in the distal-most regions of the future pupal and adult male horn. In contrast, in female *O. nigriventris* (*right*), DLL is expressed in a *more posterior region* of the prepupal horn. Unlike in males, females reabsorb a large portion of their prepupal horn during the pupal stage and develop into adults that express only a pronotal ridge (see Figs. 1 and 2). *Bottom*: In contrast, both male (*left*) and female (*right*) *Onthophagus taurus* express DLL only in a small domain at the posterior base of the prepupal thoracic horn (*highlighted by arrows* and *insets*). In this species, both sexes radically remodel thoracic horn expression via resorption of the entire horn during pupal development and molt into adults that lack any indication of a prothoracic outgrowth (see Figs. 1 and 2). Location of DLL expression, therefore, appears to covary with the degree of secondary horn tissue loss through pupal resorption, with a more posterior expression possibly designating a relatively larger fraction of the prepupal and pupal horn for subsequent retraction

Kaufman 2005). Interestingly, low levels of expression were also observed outside those proximal domains and through most of the prepupal dorsal epidermis. Two other patterning elements, DAC and EXD, did not exhibit obvious and consistent differential expression during prepupal horn development, and instead, were expressed rather ubiquitously throughout the horn primordium and adjacent areas. All three patterning elements also exhibited no obvious differences in their expression depending on species or sex.

However, a fourth patterning element, DLL, exhibited such differences. DLL expression occurred in the future distal-most region of adult thoracic horns in male *O. nigriventris*. In female *O. nigriventris*, DLL expression occurred over a similarly large domain which was located far more posteriorly. This domain did not correspond to the distal region of the future pupal horn, but more likely corresponded to the future distal-most region of the final adult thoracic ridge present in females but not in males. This raises the possibility that even though DLL is expressed in a far more posterior domain in prepupal female *O. nigriventris*, it may pattern the same region of the resulting adult structure as in males; i.e., which region of the prepupal pronotal epidermis will give rise to the distal-most adult pronotal epidermis. In addition, this may designate prepupal epidermis anterior to this proximal DLL domain for subsequent pupal retraction. This hypothesis received further support through the study of DLL expression in the thoracic horn of *O. taurus*. In this species, both sexes grow a thoracic horn during the prepupal stage, but unlike the other two species, both female and male *O. taurus* resorb their horn before the adult molt. Furthermore, while pupal horn resorption in *O. nigriventris* or *O. binodis* is incomplete and leaves behind a noticeable prothoracic ridge in adult females, pupal horn resorption in *O. taurus* is more extreme in both sexes and results in a relatively smooth, concave, adult prothorax lacking major ridges or other indications of the former presence of a thoracic outgrowth (Moczek, in review). The pattern of DLL expression mirrored the extreme, sex-uniform behavior of the pupal horn. DLL expression in the prepupal thoracic horn primordia of both female *and* male *O. taurus* was confined to a small domain at the extreme posterior base of the outgrowth.

This result is in contrast to earlier observations that argued that DLL expression is entirely absent from pronotal horns in *O. taurus* (Moczek and Nagy 2005). Instead, we know now that DLL is, indeed, expressed in pronotal horns, but restricted to a small medial domain with a width of approximately 60 μm, which corresponds to two to three sections only, and the small size of this domain may explain why it remained undetected in previous attempts. Similar to female *O. nigriventris*, the location of DLL expression in *O. taurus* clearly does not correspond to the distal region of the future pupal horn, but more likely corresponds to the future distal-most region of the final adult prothorax. As in *O. taurus*, females *and* males resorb their prepupal thoracic horn, this further supports the hypothesis that a more proximal–posterior DLL expression is associated with a greater degree of secondary horn tissue loss through pupal resorption.

This also suggests that in species such as *O. taurus* or the females of *O. nigriventris*, the proximodistal axis of the pupal and adult horn are not exactly identical, but that instead, the distal-most portion of the pupal horn far exceeds that of the adult. If this is correct, it may suggest that during prepupal development, separate and at least, partly independent patterning mechanisms may be specifying the proximodistal axis of future pupal and adult horns. While this is clearly in need of further study, preliminary evidence in support of this notion comes from an earlier

study implicating epidermal growth factor receptor (EGFR) signaling in horn differentiation. For example, *aristaless (al)* is an important target of EGFR signaling, and together with other EGFR signaling factors, it has been shown to pattern P/D axis formation in the *Drosophila* antenna and tarsal region (Schneitz et al. 1993; Campbell 2002). Additionally, the *al* ortholog in crickets is expressed in the distal portions of developing legs, mouthparts, and antennae as well as cerci (Miyawaki et al. 2002). Interestingly, *al* is also expressed strongly in prepupal thoracic horns of *O. taurus* and *O. nigriventris* (Moczek and Nagy 2005). Unlike *Dll*, however, the domain of *al* expression always corresponded to the distal most region of the future *pupal* horn and did not differ between prepupae of the two species, or sexes within both species, regardless of the degree to which their horn primordia were subsequently resorbed during the pupal stage (Moczek and Nagy 2005).

In sum, our results suggest that the origin of beetle horns relied, in part, on the redeployment of several traditional and conserved appendage patterning elements. Conversely, our results also indicate that certain aspects of horn patterning such as the exact position and domain size of DLL expression are surprisingly evolutionarily labile and may provide important avenues for the modulation of horn size and degree of sexual dimorphism in at least some species. If correct, such mechanisms may be capable of generating developmental divergences on the level of populations or closely related species well beyond what has generally been considered possible. Additional gene expression studies and the development of functional approaches are clearly necessary to further corroborate these observations.

The developmental basis of beetle horn diversity

Our results implicate the same set of four appendage patterning genes in the development of horns in three congeneric beetle species, consistent with a single origin of thoracic horns among these three species. This is at odds with recent phylogenetic analyses of the genus, which proposed three independent origins of thoracic horn development among the same three species (Emlen et al. 2005a,b). However, this phylogenetic assessment was based only on adult morphologies and did not take into account the widespread occurrence of transient thoracic horns that are resorbed during the pupal stage, and are, therefore not apparent in adults (Moczek, in review).

Our results also indicate that at least two patterning genes, *exd* and *dac*, which are known to pattern proximal and medial appendage identity in a wide range of arthropod appendages, are more or less ubiquitously expressed throughout the prepupal epithelium. A third patterning element typically involved in patterning proximal appendage identity, *hth*, exhibited strong expression in proximal appendage compartments including the horn, but low levels of *hth* expression were also commonly observed throughout the dorsal epidermis of the pronotum and head. Only *Dll* expression was confined strictly to those regions that gave rise to the distal-most region of the adult prothoracic horn. This suggests that the dorsal epidermis of the head and pronotum of *Onthophagus* beetles may be developmentally preadapted to produce outgrowths in a wide range of locations, as the only patterning element missing to complete the minimum network required for correct P/D axis formation is *Dll*.

Thus, any changes in the upstream regulation of *Dll* expression that would lead either to a novel domain of expression, or shift of the exact location of an already existing domain, would have the potential to bring about the formation of an outgrowth in a new location. Interestingly, *Onthophagus* beetles have become a textbook example for their extreme diversity in the exact location of horn expression (reviewed in Emlen 2000; Moczek 2005, 2006a,b). Horns frequently protrude from the tip, center, or lateral periphery of the head and thorax, and different species can express single or paired horns and combinations of horn types on different body regions. It is intriguing to speculate whether the radiation of horn types within the genus *Onthophagus* has been made possible through ubiquitous activation of all but one of the major p/d patterning genes during prepupal epithelial differentiation. Experiments are underway to explore expression patterns and functions of these and other patterning elements in several beetle genera that lack horns to further explore this hypothesis.

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