

WHEN ONTOGENY REVEALS WHAT PHYLOGENY HIDES: GAIN AND LOSS OF HORNS DURING DEVELOPMENT AND EVOLUTION OF HORNED BEETLES

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Abstract.—How ecological, developmental and genetic mechanisms interact in the genesis and subsequent diversification of morphological novelties is unknown for the vast majority of traits and organisms. Here we explore the ecological, developmental, and genetic underpinnings of a class of traits that is both novel and highly diverse: beetle horns. Specifically, we focus on the origin and diversification of a particular horn type, those protruding from the pronotum, in the genus *Onthophagus*, a particularly speciose and morphologically diverse genus of horned beetles. We begin by documenting immature development of nine *Onthophagus* species and show that all of these species express pronotal horns in a developmentally transient fashion in at least one or both sexes. Similar to species that retain their horns to adulthood, transient horns grow during late larval development and are clearly visible in pupae. However, unlike species that express horns as adults, transient horns are resorbed during pupal development. In a large number of species this mechanism allows fully horned pupae to molt into entirely hornless adults. Consequently, far more *Onthophagus* species appear to possess the ability to develop pronotal horns than is indicated by their adult phenotypes. We use our data to expand a recent phylogeny of the genus *Onthophagus* to explore how the widespread existence of developmentally transient horns alters our understanding of the origin and dynamics of morphological innovation and diversification in this genus. We find that including transient horn development into the phylogeny dramatically reduces the number of independent origins required to explain extant diversity patterns and suggest that pronotal horns may have originated only a few times, or possibly only once, during early *Onthophagus* evolution. We then propose a new and previously undescribed function for pronotal horns during immature development. We provide histological as well as experimental data that illustrate that pronotal horns are crucial for successful ecdysis of the larval head capsule during the larval-to-pupal molt, and that this molting function appears to be unique to the genus *Onthophagus* and absent in the other scarabaeine genera. We discuss how this additional function may help explain the existence and maintenance of developmentally transient horns, and how at least some horn types of adult beetles may have evolved as exaptations from pupal structures originally evolved to perform an unrelated function.

Key words.—Evolvability, evolutionary novelty, exaptation, horned beetle, modularity, lability, *Onthophagus*.

Received May 3, 2006. Accepted August 15, 2006.

How novel morphological traits originate and diversify continues to represent a major frontier in evolutionary biology (Raff 1996). It is generally believed that for major evolutionary innovation to occur, ecological opportunity must be met with the developmental capacity to generate novel traits as well as the genetic conditions necessary to maintain a novel trait across generations. However, exactly how ecological, developmental, and genetic mechanisms interact in the genesis and subsequent diversification of morphological novelties is unknown for the vast majority of traits and organisms (Minelli 2003). Consequently, our understanding of the biological requirements and mechanisms underlying the origins of diversity remains poor, despite this being one of the major foci of evolutionary biology since its inception (West-Eberhard 2003). Here we explore the interactions between ecological, developmental, and genetic mechanisms underlying a class of traits that is both novel and highly diverse: beetle horns.

Beetle horns are rigid cuticular outgrowths from body regions such as the dorsal head and prothorax which generally do not produce such outgrowths in insects. Consequently, the horns of beetles lack obvious homology to other structures in insects or arthropods (reviewed in Moczek 2005, 2006a). Meanwhile, relative investment into these structures is often substantial, resulting in the production of structures that regularly exceed the weight of other body parts such as legs or wings, and in some extreme cases account for more than 30% of body mass (Mizunuma 1999). Regardless of exact location

or size, all beetles studied thus far use their horns as weapons in male combat over access to females, and their functional significance and fitness consequences have been well demonstrated in a subset of species (reviewed in Emlen 2000; Moczek 2006a,b). Beetle horns can therefore be considered an example of an evolutionary novelty that has provided their bearer with a significant new phenotype: a powerful weapon in male-male competition. At the same time beetle horns are inordinately diverse. At least six families of beetles express horns, which are most diverse and elaborate in the chafers, or scarab beetle family (Arrow 1951). Here, several thousand species express horns and horn expression is highly variable both above and below the species level. Horns can develop from a variety of body regions and are typically sexually dimorphic in expression. Furthermore, in a large number of species horn expression in the male sex is discontinuous, resulting in the development of intrasexual alternative male morphologies that rely on alternative reproductive tactics to acquire matings (Emlen 2000). Consequently, dramatic diversity in size and location of horn expression is not only observed among distant families or genera of horned beetles, but commonly exists within genera or even species. Here we focus on the development and evolution of horns produced by the dorsal region of the first thoracic segment, or pronotum. In particular, we focus on pronotal horn expression in the genus *Onthophagus*, which recently emerged as a particularly promising model system for integrating developmental, genetic, and ecological perspectives on phenotypic diversification.

The genus *Onthophagus*, with over 2400 extant species, is among the most speciose genera of the animal kingdom (Balthasar 1963; Matthews 1972; Howden and Young 1981; Storey and Weir 1988, 1990). *Onthophagus* beetles are true dung beetles, cosmopolitan, and famous for their dramatic diversity in horn expression (Arrow 1951; Emlen 2000). Two recent phylogenies permit behavioral (Villalba et al. 2002) and morphological diversity (Emlen et al. 2005) to be considered in a phylogenetic context. The most recent of these analyses (Emlen et al. 2005) was specifically aimed at reconstructing the origin of horn expression in the genus *Onthophagus*, and to estimate the frequency of repeated, independent origins of horns in different parts of the genus. For example, Emlen et al. (2005) estimated a minimum of nine independent gains of pronotal horns in males and seven in females among just 48 *Onthophagus* species. Here we explore in more detail the developmental basis of pronotal horns in a variety of *Onthophagus* species as well as one species that belongs to the sister genus *Oniticellus*. We show that within the genus *Onthophagus* far more species possess the ability to develop pronotal horns than is indicated by their adult phenotypes. We then investigate the developmental and ecological reasons for these surprising observations, and explore how this alters our understanding of the origin of morphological diversity in the genus *Onthophagus*.

The Developmental Basis of Beetle Horns

Similar to the more traditional appendages of holometabolous insects the horns of adult beetles are the product of two basic developmental processes: a prepupal growth phase relatively late in larval development, followed by a pupal sculpting and remodeling phase just prior to the final, adult molt (Moczek and Nagy 2005; Moczek et al. 2006). During the prepupal stage near the end of larval development, all larval epidermis apolyses, or detaches, from the larval cuticle and selected regions undergo more or less dramatic cell proliferation to generate the pupal precursors of adult structures. The precursors of adult horns originate during this prepupal growth phase just like the precursors of adult legs, mouthparts, wings, or antennae of most insects. (The only deviation from this pattern occurs in the higher flies and the wings of Hymenoptera, Lepidoptera, and some Coleoptera where appendages originate from early developing imaginal discs, which represents a derived mode of appendage formation absent in the majority of insects (Svacha 1992)). Furthermore, recent genetic work shows that prepupal horn growth appears to be regulated by many of the same patterning genes and processes responsible for the formation of more traditional insect appendages (Moczek and Nagy 2005; Moczek 2006; Moczek et al. 2006). At the end of the prepupal stage the animal molts into a pupa, and structures that grew during the prepupal growth phase are now free to expand and become visible externally. Because adult appendages undergo most of their growth during the prepupal stage, pupae of all holometabolous insects including beetles are already equipped with a full complement of adultlike appendages. The pupal stage then marks the onset of the second developmental phase important for the expression of adult appendages, including beetle horns. During the pupal stage the pupal epidermis

apolyses once more, but instead of the rapid growth marking earlier stages, apolysis is followed by more or less subtle sculpting of the pupal epidermis into the final adult shape (Nijhout 1991; Heming 2003). The biology of sculpting has been particularly well studied in *Drosophila* and is achieved in most part through the selective removal of cells via apoptosis and autophagy-mediated programmed cell death (PCD; White et al. 1994; Cullen and McCall 2004; Martin and Baehrecke 2005). However, preliminary studies on *Onthophagus* beetles indicated that, with respect to pronotal horn development, PCD-mediated sculpting can be rather dramatic and capable of removing large amounts of pupal horn tissue over a period of just a few days, which in extreme cases allows fully horned pupae to molt into entirely hornless adults (Moczek 2006c; T. Kijimoto, A. Moczek, and J. Andrews, unpubl. data). In summary, similar to traditional appendages in holometabolous insects, the expression of horns in adult beetles necessitates an explosive prepupal growth period, resulting in the presence of adultlike horns in the pupae, and a pupal remodeling and sculpting phase prior to the final adult molt.

Focus of the Present Study

Here we survey species available to rearing in the laboratory for evidence of transient expression of pronotal horns. We then use our data to expand Emlen et al.'s (2005a) phylogeny and explore how the widespread existence of developmentally transient horns alters our understanding of the origin and the dynamics of morphological innovation and diversification in the genus *Onthophagus*. Lastly, we propose a previously undescribed function for pronotal horns during immature development and provide histological as well as experimental data to examine this function in and outside the genus *Onthophagus*. We discuss how this additional function may help explain the existence and maintenance of developmentally transient horns, and how the horns of adult beetles may have evolved as an exaptation from pupal structures originally evolved for an unrelated function.

METHODS

Species Choice and Husbandry

We studied pronotal horn development in the laboratory in seven *Onthophagus* species (*Onthophagus binodis*, *O. gazella*, *O. hecate*, *O. nigriventris*, *O. pennsylvanicus*, *O. sagittarius*, and *O. taurus*) and one species in the closely related genus *Oniticellus* (*Oniticellus militaris*). All morphological and molecular phylogenies of the Scarabaeidae published to date place *Oniticellus* very close to *Onthophagus*, and a subset of these phylogenies consider them sister taxa (e.g., Zunino 1984; Villalba et al. 2002). We included *Oniticellus militaris* in our study to explore which, if any, components of pronotal horn development may be unique to *Onthophagus* beetles, and which might be shared across multiple scarabaeine genera. *Onthophagus gazella*, *O. nigriventris*, *O. sagittarius*, *O. binodis*, and *Oniticellus militaris* were collected from field populations in Oahu, Hawaii during 2004 to 2006 and reared in the laboratory as described in Moczek and Nagy (2005). *Onthophagus taurus* was collected from pastures in Monroe

County, Indiana during the same time interval and reared similarly. *Onthophagus hecate* and *O. pennsylvanicus* were collected from pastures in Durham County, North Carolina in 2001 and reared as described for *O. hecate* in Moczek and Nijhout (2002b). We also included in our analyses descriptions of pupae of two species that we did not rear ourselves. Descriptions of pupal *O. nuchicornis* were obtained from Rosenhauer (1882) and Burmeister (1930), and descriptions of pupal *O. acuminatus* were obtained from D. J. Emlen (pers. comm. 1996, 2003).

Ancestral State Reconstructions

To explore the consequences of transient horn expression for determining ancestral character states in *Onthophagus* beetles we reconstructed ancestral states on the phylogeny presented in Emlen et al. (2005a; identical species relationships and branch lengths) using maximum-likelihood methods with Mesquite (Maddison and Maddison 2005). We assigned values to all taxa according to presence (1) or absence (0) of pupal horns. Species were scored as having pupal pronotal horns when we observed such horns during a species' ontogeny, or pupal horns had unambiguously been documented elsewhere. We also scored species as having pupal pronotal horns when pronotal horns were present in adults, even though in some of these species descriptions of pupal morphologies were not available. Scoring species in this fashion is justified because adult horns, just like adult legs, mouthparts, antennae, wings, genitalia, etc. are grown during the prepupal stage and thus their precursors must be present in the pupa. During the pupal stage horns, just like any other appendage or outgrowth, cannot be grown and can only be resorbed (Moczek 2006a). All remaining species (which did not express pronotal horns as adults and for which pupal data was not available) were conservatively scored as lacking pupal horns. It is possible that many more, if not all, species included in the phylogeny have pupal horns. We then estimated both the parsimony and likelihood ancestral states. For the likelihood analysis, we used a one-parameter (rate of change) Markov model with equiprobable loss and gain of horns. We repeated the analysis using the asymmetrical Markov k-state two-parameter model to allow for forward and reverse rates to differ. Because we are comparing nonnested hypotheses, we are unable to use the likelihood-ratio test to calculate a *P*-value for a given state at each node. Instead, weighted likelihoods for each node were subjected to a decision threshold of 2.0 log-likelihood units to make character state decisions (Edwards 1972; Pagel 1999). This cutoff is generally viewed as conservative, and has been used in a number of similar analyses (e.g., Mooers and Schuller 1999; Mast et al. 2006). The methods were applied to pupal horn data for both males and females, and adult horn data as presented in Emlen et al. (2005) for comparison.

Histological Observations

To explore possible functions of transient pronotal horns during immature development we studied the larval-to-pupal molt of *O. binodis* using two approaches. First, we observed individuals of both sexes during the molt under a dissecting scope. We observed five individuals of each sex, and doc-

umented nature and timing of developmental events. We then observed additional individuals of *O. taurus*, *O. nigriventris*, and *Oniticellus militaris* to determine whether molting sequences differed across species or genera. Secondly, we employed histological approaches to examine the behavior of individual tissue regions in more detail. Late prepupal *Onthophagus binodis*, *O. nigriventris*, *O. taurus*, and *Oniticellus militaris* were killed and tissue integrity preserved using tissue fixative as described in Moczek and Nagy (2005). Specimens were then cryosectioned, counterstained with DAPI (4'-6-Diamidino-2-phenylindole) to highlight cell nuclei and to distinguish different tissue regions and types, mounted and viewed under a fluorescence microscope.

Ablations

To test for a possible function of the prepupal pronotal horn during molting we electrosurgically ablated the epidermal tissue region responsible for producing the prepupal pronotal horn of *O. binodis*. Ablations were conducted on mid-third instar larvae ($n = 36$) approximately five days before larvae would purge their gut and enter the prepupal stage. Ablations were executed by applying brief (<1 sec) electric pulses (visible as voltage arcs) using a Promed Hyfrecator 2001 (ConMed, Utica, NY). We applied 3–5 such pulses until approximately 0.5 mm² of medial larval pronotal epidermis was ablated. Ablations were conducted while larvae were monitored under a dissecting scope. As an immediate response the ablation treatment caused the cuticle of the treated region to scar and the underlying epidermal cells to visibly coagulate. Larvae reacted to the treatment immediately in an agitated manner but typically resumed feeding within minutes after being returned to their well. Larvae were monitored daily until the pupal molt. Immediately after the pupal molt we examined the success of pronotal horn ablations by visual inspection and recorded the degree to which individuals had successfully shed their larval head capsules. Results were compared to an untreated control group ($n = 28$) as well as a group of larvae which received sham treatment by ablating a similarly large area of the ventral abdomen ($n = 35$). We repeated this experiment on *O. gazella* ($n = 15$), which naturally produces a much smaller pupal pronotal horn in both sexes, and on *Oniticellus militaris* ($n = 22$), which produces no obvious pupal horn in either sex. As with *O. binodis* results were compared to an untreated control group ($n = 24$ for both species), however, no sham surgery was conducted in these other two species.

RESULTS

Comparative Pupal Development

All nine *Onthophagus* species examined in the present study grew a medial pronotal horn regardless of sex and the degree to which the horn was retained to the adult stage (see Fig. 1 for examples for which photographs of pupae and corresponding adults are available). Male *O. nigriventris* (Fig 1A), *O. hecate* (not shown), and *O. binodis* (Fig 1B) largely retained the pupal horn to the adult, whereas the corresponding females did not. This phenomenon was reversed in *O. sagittarius* (Fig 1C), in which following a sexually mono-

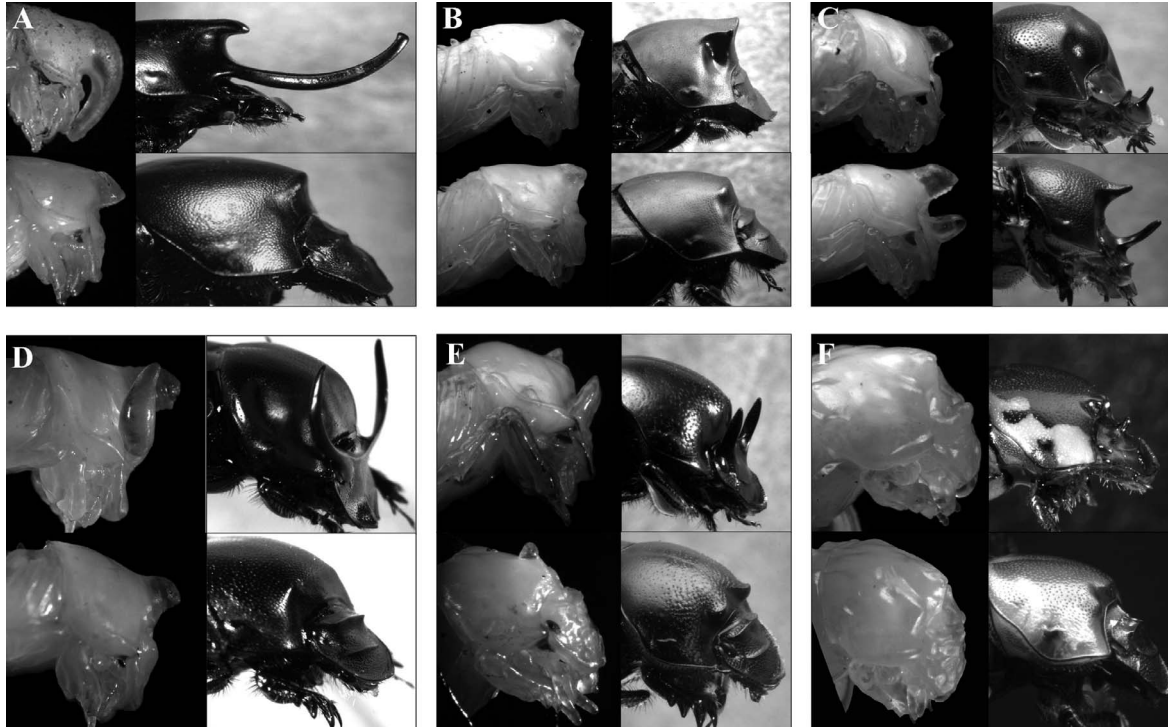


FIG. 1. Examples of horn resorption during the pupal stage. Shown are lateral views of male (top) and female (bottom) pupae and corresponding adults. In (A) *Onthophagus nigriventris* and (B) *O. binodis* only males retain their pronotal horn into adulthood while females resorb their horn almost entirely. This process is sex-reversed in (C) *O. sagittarius*, in which females, but not males, retain their pronotal horn. In (D) *O. taurus* both males and females grow a conspicuous pronotal horn but unlike in the previous species both sexes resorb their horn prior to molting into the adult. This also occurs (E) in *O. gazella* except pronotal horns in this species are much shorter compared to any other *Onthophagus* species examined thus far. (F) Similar pupal pronotal horn expression and resorption is absent in *Oniticellus militaris*, a member of the presumed sister genus to *Onthophagus*.

morphic growth phase, females retained their pronotal horn, whereas males did not. *Onthophagus gazella* (Fig 1E), *O. pennsylvanicus* (not shown), *O. taurus* (Fig 1D), *O. nuchicornis* (Burmeister 1930), and *O. acuminatus* (D. J. Emlen, pers. comm. 1996, 2003) exhibited yet another behavior. Similar to the previous *Onthophagus* species both sexes initially grew a pronotal horn which was clearly visible in all pupae examined. However, in these species both sexes subsequently resorbed their pronotal horn and molted into thorax-hornless adults without exception. Similar pupal pronotal horn expression and resorption was absent in *Oniticellus militaris* (Fig. 1F), a member of the presumed sister genus.

Phylogenetic Analyses

We compared ancestral state reconstructions to estimate the difference between results obtained by using developmental and adult data (Fig. 2A,B). We find that the ancestral state reconstruction (and the average weighted likelihood that horns are present at internal nodes) using pupal data is significantly different from the reconstruction using adult data (Fig. 2A,B; Wilcoxon signed rank test, $P < 0.0001$). Specifically, the weighted likelihood that the ability to develop thoracic horns existed at internal nodes in the pupal phylogeny was 17.9% higher than the adult phylogeny on average. Applying a likelihood decision threshold of 2.0 (so that two log-likelihood units is the minimum difference that is considered significant), we established whether horn develop-

ment occurred, did not occur, or was indeterminable at internal nodes given the terminal character states. Of the 47 nodes, 19 that decidedly lacked horn development on the adult reconstruction are now uncertain (given the data, support for each state is equal). One node state changed from definitively having horns (adult) to being uncertain in the pupal ancestral character state reconstruction. The one parameter (rate of change) model did not differ significantly from the two parameter model (likelihood ratio test: $\Delta \log\text{-likelihood} = 0.17$ (pupal), 0.1 (adult); $P > 0.1$). Furthermore, we found no character state changes between nodes on pupal male and pupal female character state reconstructions (Fig. 3; Wilcoxon signed rank test, $P = 0.82$). Instead, we found that in two of the three species postulated by Emlen et al. (2005) to have evolved pronotal horns in a sex specific manner (*O. hecate* and *O. binodis*), both sexes clearly possess the ability to grow a pronotal horn and merely differ in the degree to which they retain it into adulthood, which suggests that postulating the sex-specific origin of pronotal horns is likely an artifact of limiting character scoring to the adult phenotype (Fig. 3).

Lastly, we explored the consequences of our decision to code species that exhibited both a hornless adult morphology and an unknown pupal state as lacking pupal horns. We found that if even a single additional species is treated as having pupal horns, this can be sufficient to transform every node's state from clearly lacking horns to uncertainty on the pupal phylogeny. For instance, if either *O. alcyonides* or *O. fuli-*

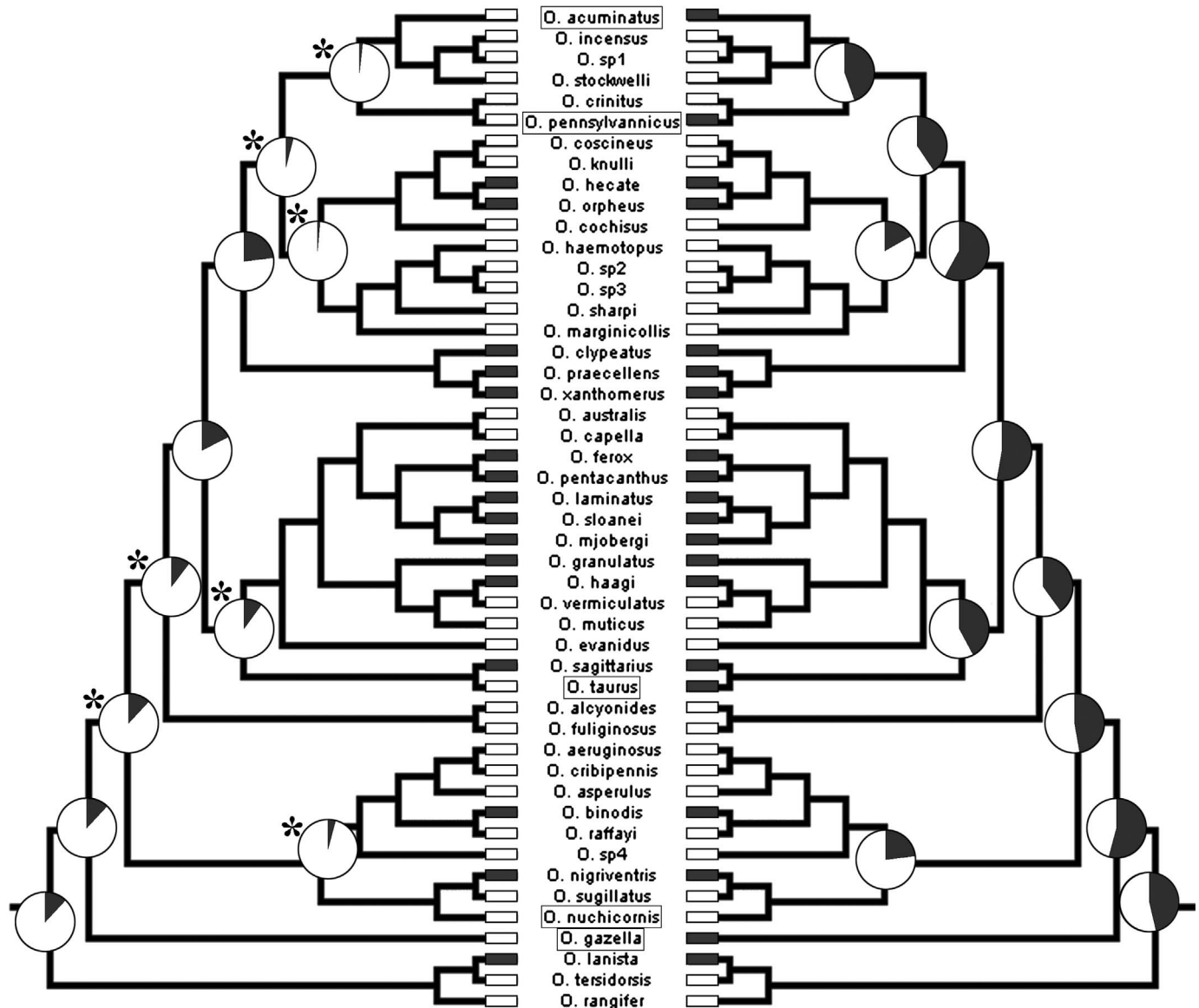


FIG. 2. Comparison of adult (left) and pupal (right) ancestral state reconstructions. Pie diagrams at each node show the weighted likelihood of horns present (black) or absent (white). Starred nodes are significant using a decision threshold of 2.0 log likelihood units. Pupal data for only five additional taxa (highlighted in boxes) are sufficient to transform 7 of 11 nodes shown (including all starred nodes) and 19 of 47 total nodes on the adult phylogeny (left) from showing a significant absence of horns to being unresolved on the pupal phylogeny (right).

ginosus actually have pupal horns, the weighted likelihood for the presence of horns increases at every node, such that all 57 nodes on the phylogeny are uncertain; it is equally possible that pupal horns represent the ancestral state. If unknown species actually have pupal horns the weighted likelihood of having horns at internal nodes can only increase.

Histological Observations

The sequence of events during the larval to pupal molt was invariable across *Onthophagus* species (Fig. 4). In each individual observed (*O. binodis*: $n = 10$; *O. gazella*, *O. nigriventris*, *O. sagittarius*: $n = 2$, respectively) the first obvious sign of ecdysis was a medial split of the meso- and prothoracic cuticle, followed by an expansion of the pronotal horn,

which, in turn, gave rise to a split of the larval head capsule along preformed suture lines. This sequence was accompanied by posterior-to-anterior convulsions of the pupal body. When viewed dorsally, haemolymph could be seen pulsing posterior-to-anterior inside the pupal pronotal horn. Haemolymph pulses tightly followed pupal convulsions, creating the impression that the animal was forcing haemolymph into the pronotal horn, thereby expanding it, which facilitated first the initial splitting and then the successful shedding of the larval head capsule (Fig. 4C,D).

Analysis of late prepupal tissue sections further supported a role of the pronotal *Onthophagus* horn in head capsule slippage (Fig. 4A,B). During mid-late prepupal development in *Onthophagus binodis*, *O. nigriventris*, and *O. taurus* the

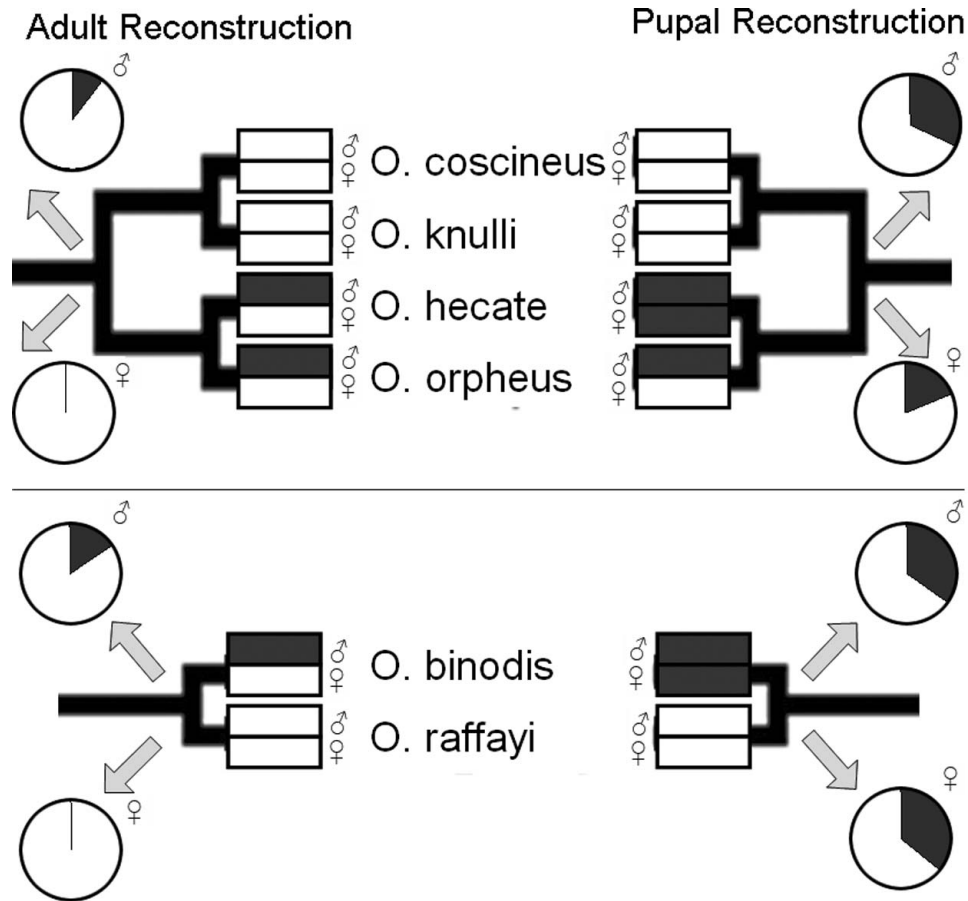


FIG. 3. Two examples of adult (left) compared to pupal (right) ancestral state reconstructions separated by sex. For each species, the top bar indicates the male character state; the bottom bar indicates the female character state. The weighted likelihoods of presence (black) and absence (white) of horns are shown for a single node in each of the two examples. Ancestral state reconstructions based only on adult morphologies suggest that pronotal horns may have originated in a sex-specific manner, whereas reconstructions based on pupal data do not.

medial anterior-most portion of the pronotal horn invariably inserted itself into the head region and expanded into the space vacated by the head epidermis after detaching itself from the cuticle approximately 48 hours earlier. In progressively later stages, increasingly lateral section also showed an advancing of the prepupal horn into the head space, suggesting that as the animal nears pupal ecdysis more and more of the space between head epidermis and cuticle is filled up by growing volume of prepupal pronotal horn tissue.

The molting sequence of *Oniticellus militaris* mirrored that of *Onthophagus* only in some aspects. Ecdysis began again with a medial split of the meso- and prothoracic cuticle which, however, appeared to be isolated from the shedding of the larval head capsule. This appeared to be achieved instead solely by inflation and expansion of the pupal head beneath, causing the larval head capsule to first split along preformed suture lines and then slip off following subsequent expansion. Sections of fixed specimen just prior to entering ecdysis further supported the notion that shedding of the larval *Oniticellus* head capsule does not involve an insertion and expansion of medial pronotal epidermis underneath the larval head capsule (not shown).

Horn Ablations

The histological observations summarized above suggested that the pronotal horn of *Onthophagus* beetles may facilitate the shedding of the larval head capsule during the larval-to-pupal molt, and that this molting function may be unique to the genus *Onthophagus* and absent in other scarabaeine genera. Experimental ablation of pronotal horn primordia further supported these hypotheses (Figs. 5 and 6). In both *Onthophagus binodis* (Fig. 6a) and *O. gazella* (Fig. 6b) ablation of pronotal horn precursor cells during larval development caused pupae to retain larval head capsules in over 80% of treated animals (*O. binodis*: $\chi^2 = 18.69$, $P < 0.001$; *O. gazella*: $\chi^2 = 13.95$, $P < 0.001$). Failure to shed larval cuticle in response to pronotal ablations was restricted to the head region only, and shedding of thoracic (the actual site of ablation) and abdominal sections of larval cuticle was unaffected. Combined, ablation of pronotal horn precursor cells resulted in pupae with typical wild-type abdomen and thorax, whereas pupal heads nearly invariably failed to eclose successfully (Fig. 5). All animals that failed to shed their larval head capsule died several days later during the pupal stage.

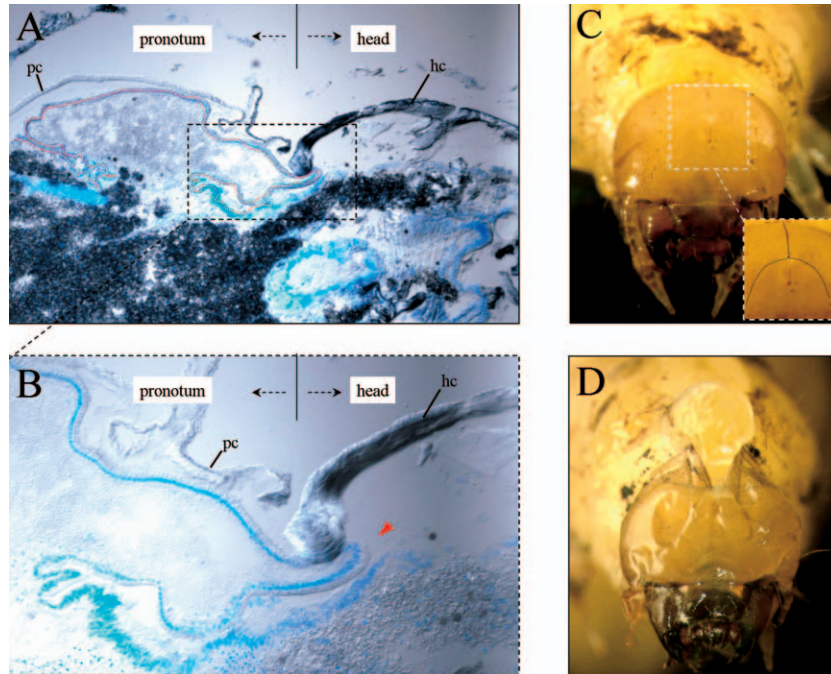


FIG. 4. The larval-to-pupal molt in *Onthophagus* involves splitting of the head capsule via internal inflation of the pronotal horn. (A) Medial sagittal section through a late prepupal *O. binodis* female. Blue staining (DAPI) highlights epidermal tissue and red dots highlight pronotal horn tissue (hc, head capsule; pc, pronotal cuticle; dorsal is up, anterior is to the right). (B) Enlargement of dorsal intersection between head and pronotum. Arrow highlights advancing tip of the pronotal horn as it becomes inserted underneath the larval head capsule. (C) Frontal view of prepupal *O. binodis* female immediately before and (D) after the pronotal horn breaks through the medial suture of the larval head capsule. Insert in (C) highlights sutures of the larval head capsule.

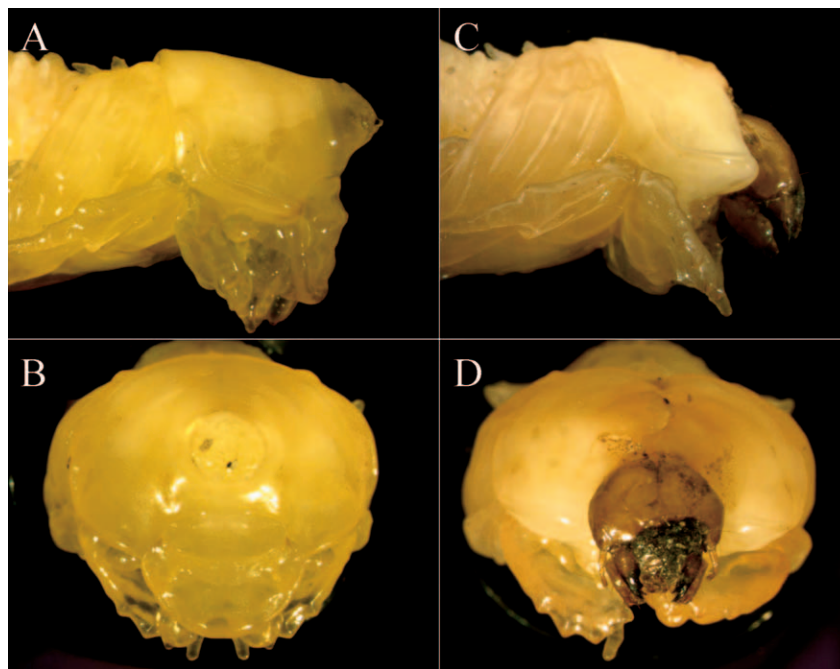


FIG. 5. Effect of larval ablation of pronotal horn precursor cells in *Onthophagus binodis*. (A) lateral and (B) frontal view of untreated control pupa. Pupa eclosed fully from the larval cuticle. (C) Lateral and (D) frontal view of pupa whose pronotal horn precursor cells were ablated during mid-larval development. The animal fully eclosed from the larval cuticle of the thorax and abdomen but failed to shed its larval head capsule.

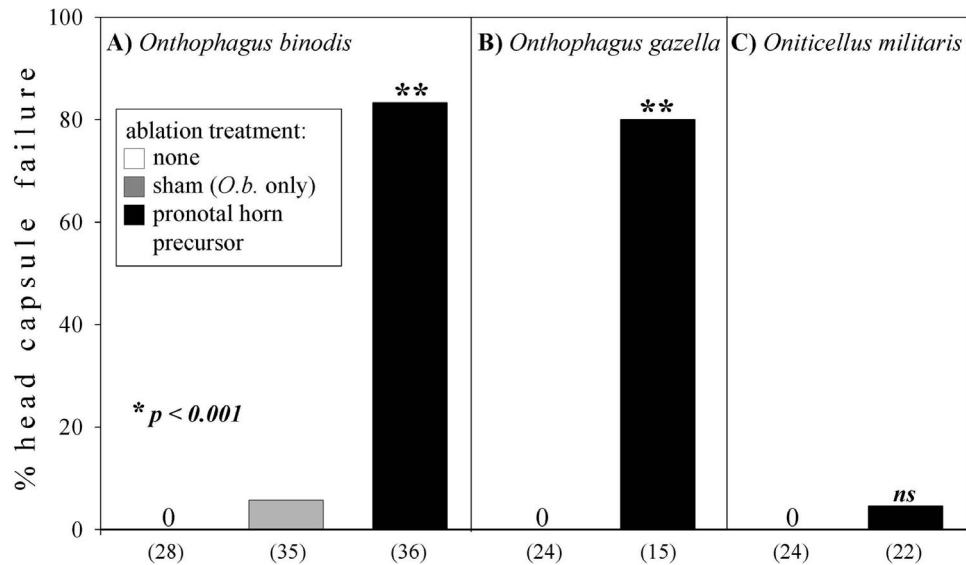


FIG. 6. Effect of larval ablation of pronotal horn precursor cells on ability to eclose from the larval head capsule during the larval-pupal molt ("head capsule failure"). (A) In *Onthophagus binodis* larval ablation (black bar) highly significantly increased head capsule failure rate compared to sham-treated (gray) and untreated individuals (0). The same effect was seen in (B) *O. gazella*, even though this species expressed the smallest pupal pronotal horn of any *Onthophagus* species thus far examined. All six *O. binodis*, and at least one of the three *O. gazella* that succeeded in shedding their larval head capsule *despite* ablation of the pronotal horn region showed signs of incomplete ablation of the pupal pronotal horn. Pronotal ablations had no effect on head capsule failure rate in (C) *Oniticellus militaris*.

All six *O. binodis*, and at least one of the three *O. gazella* that succeeded in shedding their larval head capsule *despite* ablation of the pronotal horn region showed signs of incomplete ablation of the pupal pronotal horn. Untreated control animals successfully shed their larval head capsule in all cases (both species) and only 1 of 35 sham-treated controls animals (*O. binodis* only) partially retained their larval head capsule ($\chi^2 = 1.56$, $P > 0.1$). Sham treatment did not affect ecdysis of the ventral abdomen where sham ablations were carried out. In contrast, *Oniticellus militaris* was completely unaffected by pronotal ablations, and only one of the 22 treated animals failed to completely shed its larval head capsule during pupal ecdysis (Fig. 6C; $\chi^2 = 1.07$, $P > 0.1$). Combined, these results further implicate the pronotal horn as an important structure necessary for the coordinated and complete shedding of the larval head capsule, and suggest that this function may be unique to onthophagine beetles.

DISCUSSION

Horned beetles in general, and the highly speciose and diverse genus *Onthophagus* in particular, have become promising study systems for understanding the interplay between ecological, genetic, and developmental mechanisms in the genesis of morphological diversity (reviewed in Emlen 2000; Moczek 2005). Here we use data derived from observations and experimental manipulations of immature development toward a better understanding of the origin and dynamics of adult diversification. Specifically, we explore in more detail the developmental basis of pronotal horns in a variety of *Onthophagus* species as well as one species that belongs to the sister genus *Oniticellus*. We show that within the genus *Onthophagus* transient expression of pronotal horns is widespread, and consequently, far more species possess the ability

to develop pronotal horns than is indicated by their adult phenotypes. We also show that pronotal horns carry out an important, previously overlooked, and possibly unique function during the larval-to-pupal transition of *Onthophagus* beetles. Our results have important implications for our understanding of the origin of morphological diversity in the genus *Onthophagus*, as discussed below.

Ancestral Character States

The species examined were widely distributed across the *Onthophagus* phylogeny and all possessed horns in both sexes during pupal development. Cladistic reasoning alone suggests that pupal horns are a synapomorphy of the genus *Onthophagus*. Whereas the ancestral state in the adult phylogeny was a decided lack of adult horns, our analysis using developmental data shows that at least pupal horns may represent the ancestral state in the genus *Onthophagus*. This suggests that the ability to grow an adult horn may have arisen early, because the pupal horn is a developmental prerequisite toward the expression of an adult horn. Given that we scored all taxa for which data was unavailable as lacking pupal horns, increased pupal sampling will only strengthen this result and allow us to make more definitive conclusions about the nature of both the morphological ancestral state and the evolutionary basis of the observed adult horns. Unfortunately, descriptions of pupal stages are generally rare and are likely to be impossible to obtain for many *Onthophagus* species given the rarity of some and the resistance to laboratory breeding of others. We were able to uncover descriptions of pupal *Onthophagus* morphologies for twelve more species (Table 1), none of which was included in the original phylogenetic analysis by Emlen et al. (2005), and their exact phylogenetic positions thus need to be considered uncertain.

TABLE 1. Records of pupal morphologies for twelve additional *Onthophagus* species.

Species	Reference
<i>O. amyntas</i>	Xamheu 1901
<i>O. ater</i>	Yamashita et al. 1978, 1980
<i>O. atripennis</i>	Yamashita et al. 1978
<i>O. coenobita</i>	Burmeister 1930
<i>O. fodiens</i>	Yamashita et al. 1980
<i>O. fracticornis</i>	Burmeister 1930
<i>O. latigibber</i>	F.-T. Krell, pers. comm. 2005
<i>O. lemur</i>	Goidanich and Malan 1964
<i>O. lenzii</i>	Yamashita et al. 1978
<i>O. ovatus</i>	Burmeister 1930
<i>O. ohbayashii</i>	Yamashita et al. 1978
<i>O. vacca</i>	Goidanich and Malan 1964

Interestingly, each of these twelve additional species expresses pupal pronotal horns in both sexes, and none retains their pupal pronotal horn into adulthood (see references in Table 1). Combined, and even though our sample size is still small, our results suffice to suggest that it is a reasonable, if not likely, scenario that pupal pronotal horns and thus the ability to grow a pronotal horn during prepupal development, may have arisen only a few times, and perhaps only once, during early *Onthophagus* evolution. Additional, independent support for this notion comes from recent developmental studies. Expression patterns of the limb patterning genes *Distal-less*, *aristal-less*, *dachshund*, *extradenticle*, and *homothorax* (Moczek and Nagy 2005; Moczek et al. 2006) and the Hox gene *sex-combs-reduced* (B. Kesselring and A. Moczek, unpubl. data) are highly similar in *O. nigriventris* and *O. binodis* (two species in which females resorb their pronotal horns) as well as a third species, *O. taurus* (in which both sexes resorb their horn). Emlen et al. (2005a) postulated that pronotal horns in *O. binodis* and *O. nigriventris* have originated independently, whereas the high degree of similarity in the pattern of expression of the same six genes may be more consistent with a single origin, at least among the three species examined (Moczek and Nagy 2005; Moczek et al. 2006).

Growth, Remodeling, and the Gain and Loss of Phenotypes

The horns of adult beetles originate during the prepupal stage, and as a consequence are already expressed and visible in pupae. Our examination of pupal morphologies of 21 species strongly suggest that pupal horns, and by inference the ability to grow a horn during the prepupal stage, characterize the ancestral character state of *Onthophagus* beetles. However, the expression of horns in the *adult* stage requires, in addition to a prepupal growth phase, the maintenance of horn tissue throughout the pupal stage and into adulthood. Clearly, only the pupae of some species and sexes maintain their horns, whereas others do not, and the evolutionary history and direction of horn loss during pupal development leaves room for several intriguing scenarios. For example, if pupal horns are indeed ancestral, the adult ancestral character state could still be hornless. If correct, this would imply that ancestrally, all species resorbed pupal horns before turning adult. Those species that now retain pupal horns into adulthood would have had to evolve the ability to *prevent* horn resorption, for example via interruption of the cellular and

developmental machinery employed to remove horn tissue. Importantly, such species would have *lost* the ability to resorb a pupal horn, but in turn *gained* an adult structure. Clearly, such a transition could have occurred repeatedly and independently in different lineages, and in this case we might expect to see differences in the point of interruption of the genetic and developmental network governing horn resorption in different lineages. Alternatively, the adult ancestral state could have been horned, that is ancestrally all species converted pupal into adult horns. If this is correct, those species that now resorb horns would have had to evolve the ability to initiate horn resorption during the pupal stage, for example, by recruitment of the apoptotic and autophagic cell death machinery used by all metazoan organisms to remove superfluous or damaged cells (White et al. 1994; Martin and Baehrecke 2005). Interestingly, such species would have *gained* the ability to resorb a pupal horn, but *lost* an adult structure. This transition, too, could have occurred independently in different lineages, and in this case we might expect to see differences in the endocrine and genetic regulation of horn resorption in different lineages. We are currently examining the developmental machinery underlying horn resorption and its regulation across diverse *Onthophagus* species to further explore these and related questions. Our results are thus consistent with Emlen et al. (2005a) in that multiple and independent evolutionary events are likely to have contributed to the diversity of pronotal horns among extant *Onthophagus* species. In contrast to Emlen et al. (2005), however, our results suggest that the ability to grow such structures may have originated only once in early *Onthophagus* evolution, and what truly mediated the diversification in pronotal horn expression was a given lineage's ability to retain, reshape, or fully resorb pupal horn primordia prior to the final, adult molt.

Multiple Functions of Pronotal Horns

Companion studies on the developmental genetics of beetle horns show that persisting and transient horns develop at the same time and location, utilize the same tissue regions and types, show identical prepupal and pupal differentiation patterns, and share at least qualitatively, and often quantitatively, similar expression patterns of basic appendage patterning genes (Moczek and Nagy 2005; Moczek et al. 2006). Combined, these observations leave little doubt that persisting and transient horns are homologous structures, with the only difference being that the former gives rise to a corresponding structure in the adult, whereas the latter does not. Why then do individuals invest into the growth of these structures during prepupal development, if this growth is then followed by secondary loss through pupal resorption in at least one or both sexes? Three nonexclusive hypotheses have been suggested to address this question (Moczek 2006a). Transient pronotal horns may be the result of genetic and developmental correlations between the sexes. Alternatively, transient horns may be developmental fossils retained from an ancestor that originally expressed these horns during the adult stage. Here we evaluated a third hypothesis, namely that the growth of pronotal horns may actually have an adaptive significance separate from simply generating an intermediary to

adult horns, causing selection to maintain pronotal horn development independent of whether the resulting structure is retained to adulthood or not.

Histological and in vivo observations suggested that pronotal horns aid in ecdysis of the larval head capsule. This hypothesis was strongly supported by experimental results. Over 80 % of *Onthophagus* individuals whose pronotal horn precursor cells were ablated earlier in larval development molted to pupae which lacked a pronotal horn and failed to successfully shed their larval head capsule, compared to complete molting success in untreated control animals and near complete molting success in sham-operated animals. This result was observed in both *O. binodis*, a more derived species that expresses a rather substantial pupal pronotal horn, and *O. gazella*, a species basal in the phylogeny that only expresses a small but nonetheless distinct pupal pronotal horn. However, *Oniticellus militaris*, which belongs to the presumed sister genus, does not produce an obvious pronotal horn, and pupal ecdysis was unaffected by the same pronotal ablations. Combined these data have three major implications. First, they suggest that pupal pronotal horns carry out an essential function during the larval-to-pupal transition, namely the shedding of the larval head capsule. Secondly, our data suggest that this function may be shared across many, if not all, *Onthophagus* species. Lastly, our data are consistent with the hypothesis that the molting function of pupal pronotal horns is unique to the genus *Onthophagus* and absent in other scarabaeine genera. The pupal pronotal horn in *Onthophagus* beetles would thus have an analogous function to the ptilinum of cyclorrhaphan flies, a balloon- or bladderlike organ which is everted during the emergence of the adult fly to break open the puparium, a hard pupal case derived from the last larval cuticle which serves to enclose and protect the fly during metamorphosis (Ždarek and Denlinger 1987).

Developmental Decoupling and the Recurrence of Phenotypes

Our results suggest a plausible scenario as to why many *Onthophagus* species grow pronotal horns even though those outgrowths are not used to form a functional structure in the adult. In lineages that, for whatever reason, lose horn expression in adults selection would maintain the ability to grow a pronotal horn during the pupal stage. Pupal and adult horns are therefore at least in part developmentally and evolutionarily decoupled, allowing adult horn expression to be modified, including loss of adult horns, while pupal horn expression is maintained. Pupal horns, via their molting function, may therefore act as genetic and developmental capacitors, maintaining much of the genetic and developmental machinery underlying horn development even in the absence of adult horn expression. If correct, this may facilitate the repeated recurrence and loss of adult horns in one or both sexes, ultimately resulting in an evolutionary flip-flopping between sexually monomorphic and dimorphic trait expression (West-Eberhard 2003). Even reversed sexual dimorphism could be generated in this manner, as possibly was the case in *O. sagittarius*. In this species male pupae express slightly but significantly larger thoracic horns than their fe-

male counterparts (Moczek 2006c), yet although females retain their horns into adulthood, males molt into completely thorax-hornless adults. We are currently combining comparative developmental approaches with deeper phylogenetic analyses to further disentangle the evolutionary history of the diversification of horned beetles.

Are the Horns of Adult Beetles an Exaptation?

Our results raise the interesting possibility that at least some horns types may have originated initially for reasons unrelated to the expression of a secondary sexual trait used in adult mate competition. In this scenario, pronotal horns may have evolved early in *Onthophagus* evolution to provide added power to shed the larval head capsule, and thus may have facilitated the development of more powerful head capsules. This may have been immediately adaptive as *Onthophagus* larvae, unlike the adults, feed on the tough fibrous fraction of dung provisioned by their parents, and thicker head capsules and correspondingly enhanced ability for jaw muscles to work against a stronger external skeleton may have allowed these larvae to enhance their ability to macerate their food prior to digestion (Halffter and Edmonds 1982). Regardless of the exact adaptive consequences, the production of such an outgrowth would initially only have been relevant for the larval-to-pupal transition, and removal of this structure prior to the next and last transition to the adult would likely have been important. Interestingly, pupae in the subfamily Scarabaeinae, which contains all true dung beetles including the genus *Onthophagus*, are notorious for producing simple, nonjointed projections from thoracic as well as abdominal segments (Fig. 7A,B; Burmeister 1930; Prasse 1957; Matthews 1963; Halffter and Matthews 1966; Reyes-Castillo and Martinez 1979; Halffter and Edmonds 1982; Ballerio 1999; Grebennikov et al. 2002). For example, *Oniticellus militaris* pupae express one simple medial outgrowth from both the medial meso- and metanotum, and two lateral rows of outgrowths from each abdominal segment (Fig. 7A,B). Importantly, at least the medial meso- and metanotal outgrowths of *Onthophagus taurus*, *O. nigriventris*, and *O. binodis* share expression of some of the same patterning genes used to pattern the pronotal horn, including the transcription factors *Distal-less*, *dachshund*, *extradenticle*, and *homothorax* (Moczek et al. 2006). These outgrowths are generally referred to as pupal support structures (Burmeister 1930; Ballerio 1999; Grebennikov et al. 2002), but their supporting function, if any, is entirely unknown (Halffter and Matthews 1966). Furthermore, to the best of our knowledge these pupal support structures have no obvious correspondence to any structures in the adult. Scarabaeine pupae thus are well equipped to express simple and small outgrowths in a wide range of body regions and to remove them prior to molting into the adult, and it is reasonable to assume that the onthophagine ancestor is likely to have had both of these capabilities. Evolving the ability to express similar outgrowths in the adult would thus only have required a relatively simple step: the evolutionary loss or inactivation of the developmental mechanism used to remove the outgrowth prior to the adult molt. This would have immediately resulted in the formation of a simple outgrowth in the adult. Even though such an outgrowth would

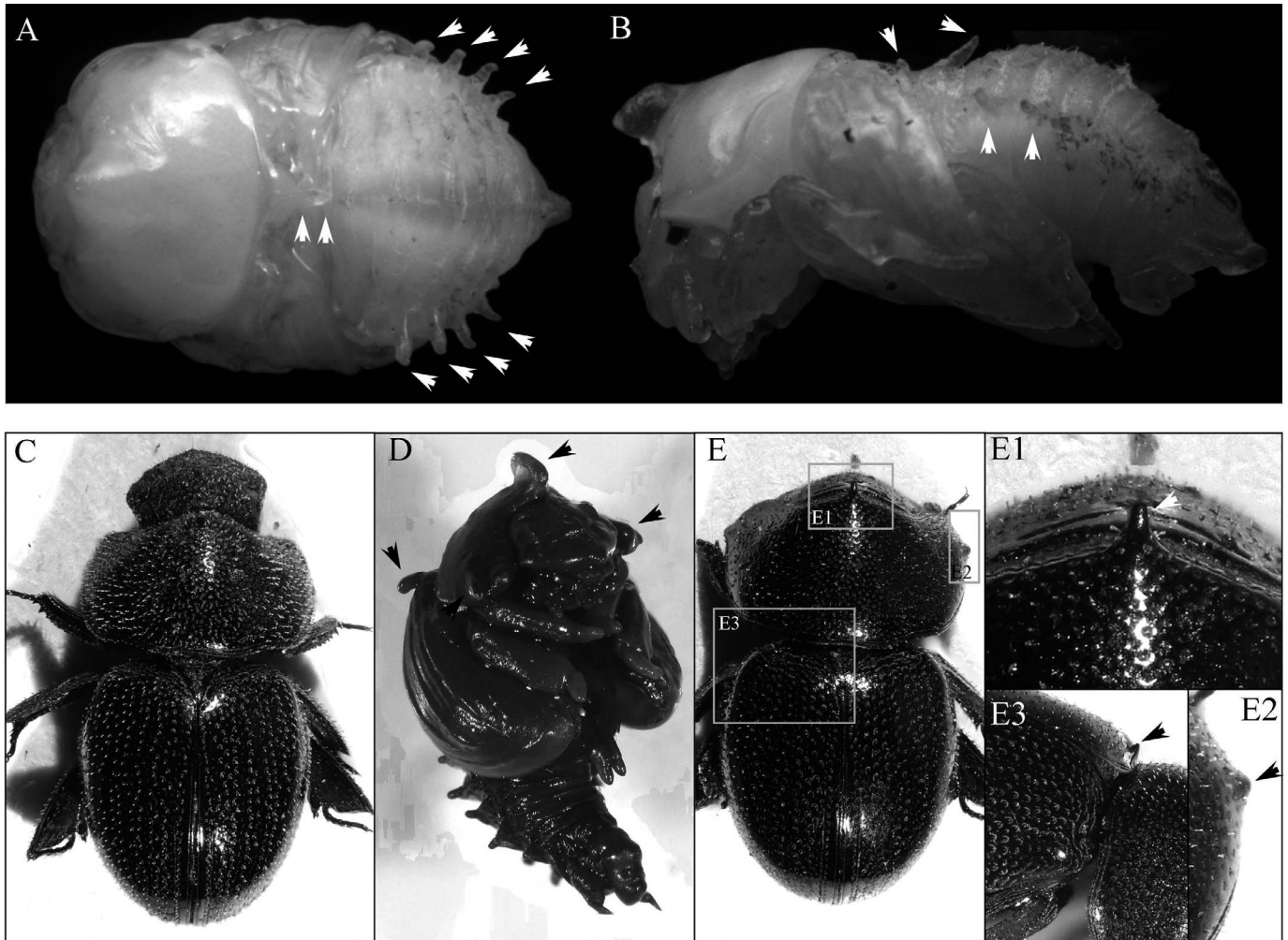


FIG. 7. Some beetle horns may derive from pupal-specific structures that originally evolved in a different context. Pupae in the subfamily Scarabaeinae frequently express simple, nonjointed outgrowth from a variety of thoracic and abdominal segments. These “pupal support structures” have no known function or counterpart in the adult phenotype. Examples shown are (A) *Oniticellus militaris* and (B) *Onthophagus binodis*. Arrows highlight lateral and medial outgrowths. (C–E) Some horns of adult beetles may have been derived from these normally pupal-specific structures via failure to remove these structures prior to the adult molt, as in the example shown here. (C) Wildtype *Pterorthochaetes insularis* normally do not express the pronotal or elytral outgrowths present in (D) the pupal stage (highlighted by arrows). (E) Developmental mutant of *P. insularis*. This individual has retained a medial pronotal “horn” (close-up in E1), symmetrical lateral protuberances of the pronotum (E2), as well as symmetrical projections off the anterior margins of both elytra (E3, here the animal was rotated to highlight the left elytral projection). Images C–E are courtesy of Alberto Ballerio.

initially most likely have been rather small we know from behavioral studies that even very small increases in male horn length carry with them significant increases in fighting success and fitness (Emlen 1997; Moczek and Emlen 2000; Hunt and Simmons 2001). Interestingly, occasional retention of pupal-specific support structures into the adult stage has been documented in a few cases (Fig. 7C–E; Paulian 1945; Ballerio 1999), including possibly one *Onthophagus* species (Ziani 1994). These anecdotal observations suggest that such retention occurs in nature at least frequently enough to be detectable by entomologists, and it is intriguing to speculate that the dramatic diversity of pronotal horns of adult *Onthophagus* may have originated initially as accidental carry-overs of pupal-specific structures that originally had evolved for an entirely different purpose.

ACKNOWLEDGMENTS

We thank D. Emlen and C. Cunningham for providing us with the *Onthophagus* phylogeny and for their help in reconstructing the original topologies. A. Ballerio generously provided photographs of *Pterorthochaetes insularis* used in Figure 7C–E. P. Nista, M. Wade, members of the Wade Lab, and two anonymous reviewers provided helpful comments on earlier versions of this manuscript. C. Jacobson, R. Kirschenbaum, and J. Micantin expertly located and collected beetles in the field. This research was carried out with support from a Howard Hughes undergraduate research fellowship to JAS, a National Science Foundation IGERT (DGE-0504627) fellowship to TEC, and a National Science Foundation Grant IOB 0445661 to APM.

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APPENDIX 1.

Weighted likelihood of horn presence at all interior nodes. Numbering of nodes in the table corresponds with numbering of each node in the phylogeny shown in Appendix 2 (identical to phylogeny presented in Figure 2). Weighted likelihoods are reported for pupal and adult males, pupal females, and the difference between pupal and adult males.

Node	Pupal male	Adult male	Pupal female	PM-AM
2	0.46250643	0.185107	0.46393891	0.27739945
3	0.54073973	0.1197706	0.54026615	0.42096911
4	0.47339971	0.1187059	0.474666153	0.35469378
5	0.39883139	0.1031311	0.400237857	0.29570034
6	0.528799853	0.1738935	0.52420953	0.3549064
7	0.58177247	0.2314925	0.57476225	0.35028001
8	0.40682845	0.0402599	0.389997252	0.36656851
9	0.444502855	0.0169762	0.43465279	0.42752666
10	0.446235649	0.0137762	0.4400666	0.4324595
12	0.08444177	0.0048553	0.08579007	0.07958647
13	0.00864327	4.85E-04	0.008987452	0.00815831
17	0.54334968	0.0149998	0.54039267	0.52834986
20	0.1679021	0.0144471	0.124572154	0.15345504
21	0.20288115	0.0371512	0.11952715	0.16573
22	0.286603799	0.1027656	0.144952653	0.18383823
23	0.05534967	0.0093582	0.04323904	0.04599146
26	0.877656755	0.9251007	0.302778755	-0.0474439
30	0.06903778	0.0039542	0.057172	0.06508355
31	0.04082506	0.0025815	0.03872269	0.0382436
32	0.025989752	0.0018426	0.02593575	0.0241472
34	3.17E-04	3.12E-05	3.33E-04	0.00028607
39	0.909756653	0.9388221	0.906455097	-0.0290654
41	0.97135675	0.9916092	0.9699084	-0.0202524
44	0.42259962	0.099221	0.420673451	0.32337867
45	0.22516203	0.0510318	0.2257598	0.17413025
46	0.26381713	0.0788061	0.26523679	0.18501107
47	0.35346556	0.1483505	0.35604355	0.2051151
48	0.17843679	0.0481068	0.18154682	0.13032994
51	0.86073305	0.914032	0.85861319	-0.05329898
52	0.99298008	0.9983369	0.99269953	-0.00535682
55	0.97175475	0.9939923	0.97057088	-0.02223751
56	0.97994755	0.9971216	0.9789523	-0.01717409
60	0.32326428	0.1536244	0.32517255	0.16963993
62	0.05548326	0.0242278	0.05610081	0.03125545
63	0.09551775	0.045088	0.09678501	0.0504298
68	0.6159783	0.1698858	0.61222554	0.44609247
71	3.92E-05	5.28E-06	4.06E-05	3.3895E-05
74	0.23038115	0.0417674	0.23665435	0.18861379
75	0.11683598	0.010598	0.12184169	0.10623797
76	0.13120905	0.0139462	0.13669675	0.11726288
77	0.13743598	0.0126625	0.14355814	0.12477345
78	0.18342793	0.024588	0.19038077	0.15883997
82	0.31203745	0.1559735	0.31652212	0.156064
86	0.24862936	0.0592839	0.25473349	0.1893455
87	0.35810553	0.1989591	0.36159915	0.1591464
92	0.04224525	0.0152868	0.04300705	0.02695841
93	0.0832627	0.037174	0.08463445	0.04608875

APPENDIX 2.

Phylogeny used to reconstruct ancestral character states in Figures 2 and 3. Numbering of nodes in the phylogeny corresponds with numbering of nodes listed in Appendix 1.

