Differential recruitment of limb patterning genes during development and diversification of beetle horns

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The origins of novel complex phenotypes represent one of the most fundamental, yet largely unresolved, issues in evolutionary biology. Here we explore the developmental genetic regulation of beetle horns, a class of traits that lacks obvious homology to traits in other insects. Furthermore, beetle horns are remarkably diverse in their expression, including sexual dimorphisms, male dimorphisms, and interspecific differences in location of horn expression. At the same time, beetle horns share aspects of their development with that of more traditional appendages. We used larval RNA interference-mediated gene function analysis of 3 cardinal insect appendage patterning genes,*dachshund*, *homothorax*, and *Distal-less*, to investigate their role in development and diversification of beetle horns within and between species. Transcript depletion of all 3 patterning genes generated phenotypic effects very similar to those documented in previous studies that focused on general insect development. In addition, we found that *Distal-less* and *homothorax*, but not *dachshund*, regulate horn expression in a species-, sex-, body region-, and body size-dependent manner. Our results demonstrate differential co-option of appendage patterning genes during the evolution and radiation of beetle horns. Furthermore, our results illustrate that regulatory genes whose functions are otherwise highly conserved nevertheless retain the capacity to acquire additional functions, and that little phylogenetic distance appears necessary for the evolution of sex- and species-specific differences in these functions.

The origin of novel features is both one of the oldest and, at the same time, one of the most poorly understood frontiers in evolutionary biology (1, 2). Although evolutionary biologists have developed powerful theoretical frameworks and experimental tools to understand the evolutionary modification of existing traits, we know remarkably little about the ecological, genetic, and developmental mechanisms—and the interactions between them—that mediate the origin and subsequent diversification of novel features. In fact, even the terminology is often misleading, and considerable debate exists regarding exactly what constitutes novelty in evolution (3). For example, Mueller and Wagner (4) define a novelty as "a structure that is neither homologous to any structure in the ancestral species nor homonomous to any other structure in the same organism." In other words, novelty starts where homology ends. At the same time, one of the most celebrated contributions of evolutionary developmental biology has been the realization that the extraordinary morphological diversity that exists on the level of organisms and their parts is not paralleled by a corresponding diversity in genetic and developmental mechanisms. Instead, the developmental genetic underpinnings of morphological diversity are remarkably conserved, and highly divergent organisms rely on the same genetic and developmental tool box to instruct the development of very different, and clearly nonhomologous, organs and structures (5–7). Inversely, a growing body of evidence shows that the opposite also occurs frequently [i.e., highly conserved and clearly homologous phenotypes diverge, at times dramatically, in their underlying developmental and genetic regulatory mechanisms, and phenomenon referred to as developmental systems drift (8) or phenogenetic drift (9)]. Exactly where homology and diversification therefore end and novelty begins becomes difficult to ascertain. Thus the origin of novel features, and the mechanisms capable of initiating innovation and elaboration of novel phenotypes from within the confines of strict homology, remain remarkably poorly understood. Here we begin to explore the developmental genetic regulation of a class of traits that is both novel and highly diverse: beetle horns (Fig. 1*A*).

Several thousand species of beetles express horns—rigid, nonjointed, and often massive projections of the exoskeleton of the head and thorax (10, 11). Horns are used as weapons in combat and are frequently sexually dimorphic (reviewed in refs. 11 and 12) (Fig. 1*A*). In a subset of species, individuals within a sex, typically males, also vary discontinuously in horn expression. In such cases, only males above a critical size threshold express horns, whereas smaller males remain female-like and hornless (Fig. 1*A*). Most importantly, beetle horns lack obvious homology to other structures in beetles or insects. They are not modified mouthparts or legs, instead they exist in addition to these structures in body regions in which insects otherwise do not produce outgrowths (13). Hence, horns can be considered an evolutionary novelty that beetles evolved at some point in their history and which fueled one of the most impressive radiations of secondary sexual traits known in the animal kingdom (11, 12).

Despite their novel nature, beetle horns nevertheless share many developmental properties with more traditional insect appendages, such as legs and antennae (14). In *Onthophagus*, the by far best-studied genus of horned beetles, the development of horns first becomes obvious during the prepupal stage at the end of larval development. At this point 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 (Fig. 1 *B* and *C*) (14). At the end of the prepupal stage the animal then molts into a pupa, and structures that grew during the prepupal growth phase are now free to expand and become visible externally. The pupal stage then marks the onset of a second developmental phase important for adult horn expression. During this stage, the pupal epidermis apolyses once more, but instead of the rapid growth marking earlier stages apolysis is followed by sculpting and remodeling of the pupal epidermis into the final adult shape. Remodeling can be subtle to dramatic, and in extreme cases is capable of removing large amounts of pupal horn tissue over a period of just a few days (Fig. 1*C*), allowing fully horned pupae to molt into entirely hornless adults (15–17). More

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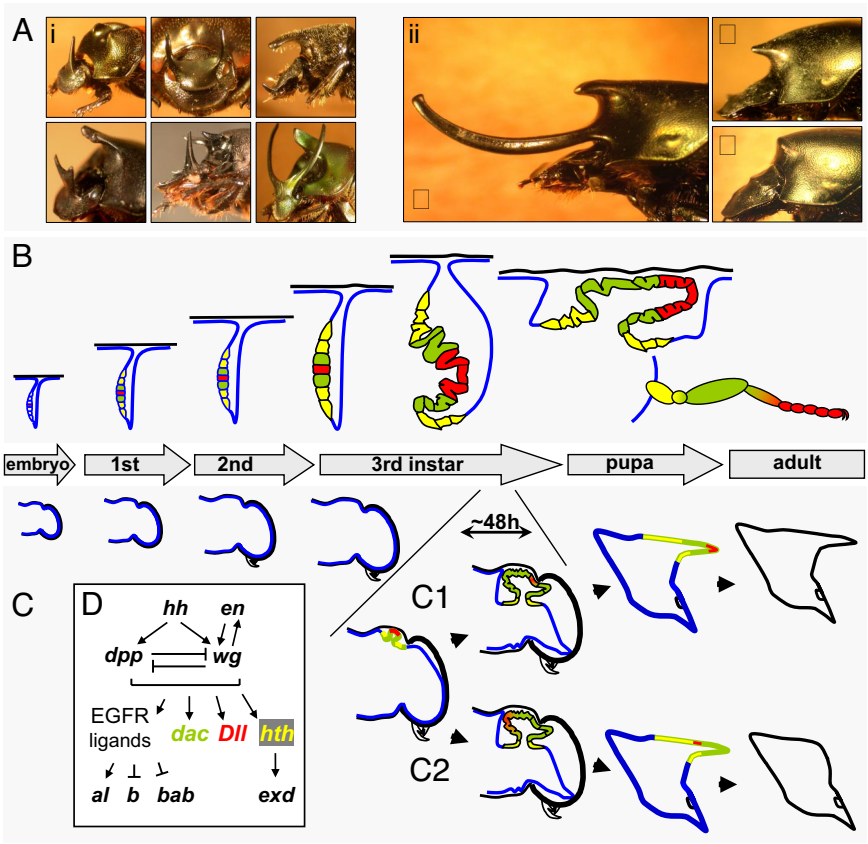

Fig. 1. Diversity and development of beetle horns. (*A*) Diversity in number, size, location, and shape of horn expression between (*i*) and within (*ii*) species of *Onthophagus*. (*B* and *C*) *Drosophila* model of limb formation (*B*) compared with the development of a thoracic beetle horn from embryo to adult (*C*). Cuticle is shown in black, epidermis in blue, including schematic expression domains of the proximodistal patterning genes *homothorax* (*hth*, yellow), *dachshund* (*dac*, green), and *Distal-less* (*Dll*, red). *Drosophila* legs develop from imaginal discs; epidermal invaginations specified during embryonic development, which grow throughout larval development. Patterning takes place while the disc is a 2-dimensional sheet of tissue, and all disc growth occurs while the disc is invaginated into the body interior. In contrast, beetle horns appear not to be specified during embryonic development. Instead, horns grow from the start as 3-dimensional epidermal outbuddings and all growth is confined to the relatively brief prepupal stage and takes place while the primordium is evaginated into the space between the epidermis and larval cuticle. In addition to a rapid prepupal growth phase, horn expression is also affected at times by a drastic pupal remodeling phase (*C1* and *C2*) during the early pupal stage. During this stage pupal horn primordia are either converted into a future adult structure (*C1*) or resorbed (*C2*) via programmed cell death. In the later case, expression of *Dll*, but not *hth* or *dac*, is shifted more posteriorly. (*D*) Position of *dac*, *hth*, and *Dll* within the basic *Drosophila* limb patterning network (*hh*, hedgehog; *en*, engrailed; *dpp*, decapentaplegic; *wg*, wingless; *EGFR*, epidermal growth factor receptor; *al*, aristaless; *b*, bar; *bab*, bric a brac; *exd*, extradenticle).

generally, beetle horns therefore originate and differentiate in a manner rather similar to the primordia of adult legs, mouthparts, wings, or antennae of most insect orders (18). The only dramatic deviation from this pattern occurs in all appendages produced by higher flies as well as the wings of Hymenoptera, Lepidoptera, and some Coleoptera, where appendages develop from imaginal discs, which represents a highly derived mode of appendage formation absent in the majority of insect orders (Fig. 1 *B* and *C*, and see later discussion). Unfortunately, most of our understanding of insect appendage formation comes from studies of imaginal disc development in *Drosophila* (reviewed in ref. 19), which derives all its adult appendages from imaginal discs (Fig. 1*B*). Imaginal discs are epidermal invaginations specified during embryonic development, which grow throughout larval development (although most growth occurs during the last instar; see Fig. 1*B*). Importantly, many important patterning steps take place while the disc is essentially a 2-dimensional sheet of tissue, and all disc growth occurs while the disc is invaginated into the body interior (Fig. 1*B*). Beetle horns differ in that they appear not to be specified during embryonic development. Furthermore, horns grow from the start as 3-dimensional epidermal outbuddings as larval horn primordia evaginate into the space between the epidermis and larval cuticle (20) (Fig. 1*C*). Consequently, the *Drosophila* model of limb development has likely limited applicability for beetle horns. However, appendage development has been studied far less outside higher flies, and consequently the *Drosophila* model of limb development retains an important reference function. Here we explore the regulation of the proximodistal axis (p/d-axis) as well as general growth of beetle horns during the prepupal growth period.

In *Drosophila* limbs, establishment of the p/d-axis begins with the concentration-dependent, combined action of 2 diffusible morphogens, *wingless* (*wg*) and *decapentaplegic* (*Dpp*), which subdivides imaginal discs into roughly concentric, nested domains of expression of several transcription factors, including *Distal-less* (*Dll*), *dachshund* (*dac*), and *homothorax* (*hth*). The center of the leg disc, characterized by *Dll* expression, eventually gives rise to the distal region of the adult appendage, whereas progressively more peripheral disc regions, characterized by *dac* and *hth* expression, form progressively more proximal appendage regions once the imaginal disc telescopes outwards to form the adult appendage (19). As highlighted above, in most other insects adult appendages do not develop from imaginal discs but via the outbudding of selected epidermal regions during late larval development (e.g., refs. 20–23). Despite these fundamental differences in appendage morphogenesis there appear to remain many similarities in the underlying patterning mechanisms. For example, *Dll* expression in the distal region and *hth* expression in the proximal region occurs during the development of appendages in a wide range of arthropods (23–29), and *Dll* activity is functionally required for distal leg formation in beetles and spiders (30, 31). *Dll*, *dac*, and *hth* therefore represent legitimate candidate genes for the regulation of p/d-axis formation and growth during beetle horn development. Furthermore, previous work has shown that all 3 p/d-axis patterning genes are expressed during the development of *Onthophagus* appendages, including horns (14, 32). Specifically, earlier expression studies in 3 *Onthophagus* species documented *dac* expression in proximal and medial, but not distal, leg regions, consistent with a conservation of patterning function during *Onthophagus* leg development (32). However, *dac* expression was ubiquitous throughout the thoracic horns of the same 3 species, suggesting that *dac* may lack a horn patterning function, or alternatively, may have changed function during the evolution of horns (32). In contrast, *Onthophagus hth* was found to be expressed in proximal and medial appendage regions of legs, antennae, and horns (32) (Fig. 1*C*), consistent with co-option and conservation of *hth* function in horn evolution and development. Last, *Onthophagus Dll* expression was found to be restricted to mediodistal portions of traditional appendages, as well as the distal portions of horns (14, 32) (Fig. 1*C1*), consistent with

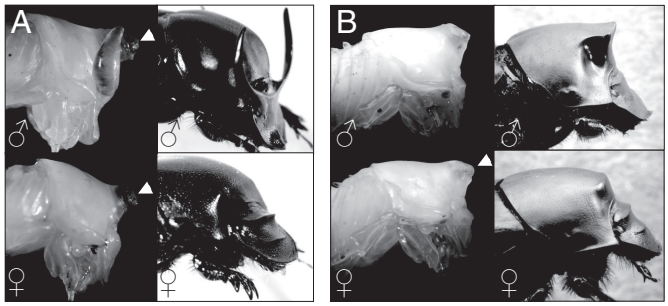

Fig. 2. Males (*Upper*) and females (*Lower*) of *O. taurus* (*A*) and *O. binodis* (*B*). Pupae are shown on the *Left* and corresponding adults on the *Right*. Arrows highlight cases of pupal horn resorption.

a conservation of *Dll* function in *Onthophagus* appendage development and a co-option of *Dll* function during horn evolution. The only deviation from this pattern was found in thoracic horns fated to be resorbed during the pupal stage, in which case *Dll* expression was shifted more posteriorly (Fig. 1*C2*)

Here we use larval RNAi-mediated transcript depletion of *Onthophagus dac*, *hth*, and *Dll* to examine their function in (*i*) *Onthophagus* development in general and (*ii*) their possible involvement in growth and p/d-axis formation of horns. We focus our efforts on 2 species, *Onthophagus taurus* and *Onthophagus binodis*, which differ in patterns of horn expression both between and within sexes. Males and females in both species grow thoracic horns during the prepupal stage, which are clearly visible in pupae (Fig. 2), but only male *O. binodis* retain this pronotal horn into adulthood. In addition, large male *O. taurus* also grow a pair of head horns, which are greatly reduced in smaller males, and completely absent in females (Fig. 2). The two species diverged from a common ancestor approximately 24 million years ago (calculated based on ref. 33). We show that all 3 patterning genes regulate the formation of traditional appendages in a highly conserved fashion, and that *Dll* and *hth*, but not *dac*, regulate horn expression in a species-, sex-, body region-, and body size-dependent manner.

Results

Cloning and Sequence Analysis. Using PCR with degenerate nested primers, we cloned partial coding sequences of *O. binodis* (*Ob*)*hth*, *O. taurus* (*Ot*)*Dll* and *ObDll* (354 bp, 312 bp, and 336 bp, respectively) from cDNA representing mixed late-larval and prepupal stages. Two discontinuous sequences of *OtDll* were cloned, including 1 upstream of the homeodomain (HD) used for RNAi studies and another encompassing the HD, which combined were used for sequence and phylogenetic analysis (Figs. S1 and S2). Cloning and sequence analysis of *O. taurus dachshund* (*Otdac*) are described in ref. 32. We obtained 30 *hth* and 25 *Dll* individual clones. All nucleotide sequences were compared with each other and to those previously described for *hth* and *Dll* in GenBank. No evidence of paralogous copies of the *hth* or *Dll* genes was found.

Sequence analyses support that these genes are the *Onthophagus* orthologs of *Tribolium castaneum hth* (*Tchth*) and *Dll* (*TcDll*) (Figs. S1 and S2). *Tchth* is predicted to encode a 456-aa polypeptide with 2 highly conserved domains, an N-terminal MEIS domain (HM), and a homeodomain (HD) located near the C terminus (34). We observed 99% amino acid and 79% nucleotide identity with *Tribolium hth* over the 354 bp *Ob* region (consisting of the nearly complete MEIS domain). The region used for RNAi is 100% identical to the corresponding *Tribolium* sequence. Sequence alignment also shows a strong degree of conservation in the HM domain known to mediate interactions with *Exd* (35). Phylogenetic reconstructions using neighbor-joining (Fig. S1) and maximum-likelihood methods group *Obhth* with *Tribolium* as expected.

TcDll is predicted to encode a 312-aa polypeptide with a highly conserved HD (30). Outside the HD, the *OtDll* (*ObDll*) sequence is 85% (86%) identical to that of *Tribolium* over the 68-aa (47-aa) cloned region. Within the HD, *OtDll* and *ObDll* are 100% and 98% identical at the amino acid level and 78% and 79% identical at the nucleotide level, respectively. In addition, there is strong conservation in several upstream motifs.

Phylogenetic analysis (Fig. S2*B*) of *ObDll* and *OtDll* shows that both sequences cluster with other coleopteran sequences (*Tribolium*, *Harmonia*). However, the exact placement of *ObDll* and *OtDll* within the Coleoptera, as well as higher-order relationships within the arthropods, are less robust as indicated by low bootstrap support at these nodes. It is our belief that overall short gene sequence lengths combined with incompleteness of sequence data across taxa in the highly variable regions outside the homeodomain contribute to the *Dll* tree being a poor indicator of species relationships. Phylogenetic reconstruction using neighbor-joining (Fig. S2) and maximum-likelihood-methods generate similar topologies.

RNAi Effects on Appendage and Horn Development. Larval RNAi-mediated transcript depletion of *Otdac* generated many phenotypes consistent with a general conservation of *dac* function during *Onthophagus* development compared with other insects. For instance, pupal and adult legs and antennae exhibited shortening, fusion, or deletion of medial appendage regions (Fig. 3*A*) similar to *dac* RNAi phenotypes described in other insects (23, 36). Also, larval *Otdac* RNAi resulted in smaller compound eyes with rough ommatidial surfaces, including ommatidial misarrangements and partial fusion of ommatidia, phenotypes similar to those observed in *Drosophila dac* mutants (Fig. 3*A*) (37). These phenotypes were observed in 81% of pupae (n = 25/31) and 89% (n = 16/18) of adults generated. However, the same individuals in which *Otdac* RNAi resulted in unambiguous *dac*-characteristic RNAi effects elsewhere in their body revealed no effects on thoracic and head horn expression (Fig. 3 *B* and *C*). Correspondingly, body size–horn length allometries did not differ between control animals and those with unambiguous *dac*-characteristic RNAi effects elsewhere in their body (Fig. 3 *B* and *C*). This observation supports the hypothesis that despite being expressed during horn development (32), *dac* lacks an obvious horn patterning function, at least in *O. taurus*.

Larval RNAi-mediated transcript depletion of *O. taurus homothorax* (*Othth*) also generated many phenotypes indicative of a high level of conservation of *hth* function during *Onthophagus* development. *hth* has been well characterized as a regulator of proximal appendage identity (19), antennal identity (38, 39), mouthpart identity (36, 39), eye differentiation (40), and as a cofactor to several Hox genes (40), including *Sex combs reduced* (*Scr*) (41). Consistent with these earlier studies, *Othth* RNAi resulted in a reduction and deformation of the coxa, the most proximal leg segment. *Othth* RNAi also resulted in premature eye differentiation evidenced by visible pigmentation of ommatidia already in late prepupae, whereas untreated individuals had to reach the third day of the pupal stage to express similar levels of pigmentation. Furthermore, *Othth* RNAi generated at least 2 striking partial to complete homeotic transformations, including antennal transformations to a mixed antenna/leg identity, and complete transformation of the hypopharyngeal sclerites, which are part of the labial-hypopharyngeal complex, to a structure closely resembling the maxillary lacinia in shape, color, and bristle patterning (Fig. 3*D*). Most spectacularly, *Othth* RNAi resulted in the expression of ectopic wing tissue on both sides of the pronotum (Fig. 3*D*). Last, *Othth* RNAi resulted in an enlargement of the prothoracic segment relative to both head and abdomen, and we therefore used body mass rather than thorax width as a general measure of body size. The above phenotypes were observed in at least 74% (n = 23/31) of pupae and adults. Importantly, *Othth* RNAi also dramatically affected thoracic horn expression and *Othth* RNAi males with unambiguous *hth* RNAi effects elsewhere in their body also exhibited much shorter thoracic

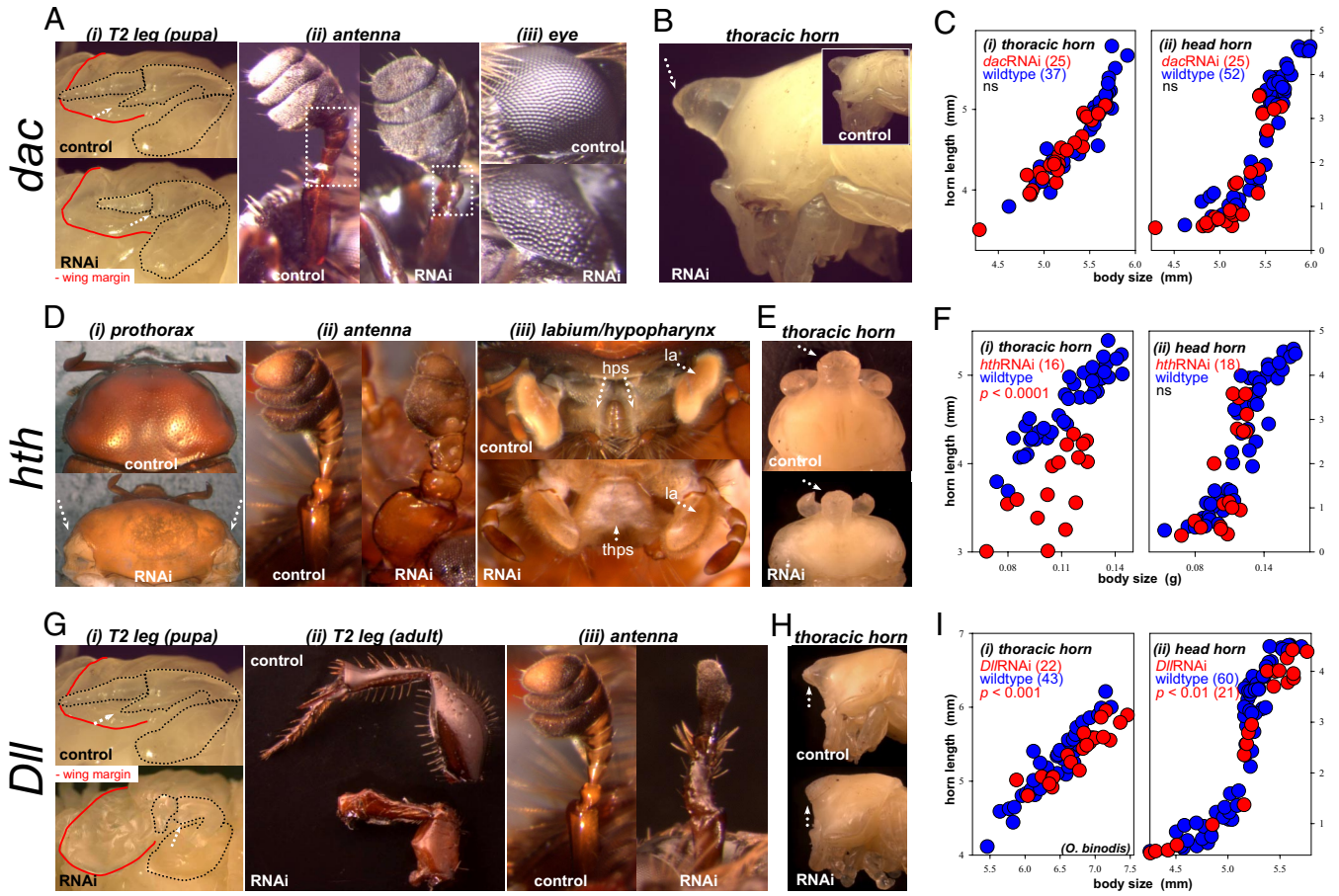

Fig. 3. Larval RNAi-mediated transcript depletion of *dachshund* (*A–C*), *homothorax* (*D–F*), and *Distal-less* (*G–I*). Images illustrate typical phenotypes observed in each experiment compared with wild-type phenotypes. Graphs depict scaling relationships between pupal body size and horn length for thoracic horns (*i*) and head horns (*ii*). Pupal body size was measured as thorax width for *dac* and *Dll*. *Hth* RNAi affected thorax shape and we therefore used pupal mass as an estimator of body size. Wild-type is shown in blue and RNAi-treated individuals are shown in red. All data are from male *O. taurus* except *Ii*, which were collected from female *O. binodis*. Sample sizes are given in parentheses.

horns than expected given their body mass ($t = 8.72$, df = 18, $P < 0.001$) (Fig. 3 *E* and *F*). At the same time, *Othth* RNAi left head horn expression completely unaffected despite dramatic transformations elsewhere in the body ($t = 0.65$, df = 21, P = not significant) (Fig. 3*F*). Instead, *Othth* RNAi males developed head horns precisely the right length given their individual body mass. These results suggest that *Othth* is involved in the regulation of at least some, but not all, horn types.

Larval RNAi-mediated transcript depletion of *Onthophagus Distal-less* (*ODll*) was similarly effective in generating many phenotypes, indicative of a high level of conservation of *Dll* function during *Onthophagus* development (19, 36). Most obviously, *Dll* RNAi resulted in a loss or fusion of distal appendage regions in pupal and adults legs, mouthparts, and antennae (Fig. 3*G*). Knockdown phenotypes were observed in at least 85% ($n = 52/61$) *O. taurus* pupae and adults injected with *dsOtDll* and all 56 *O. binodis* pupae and adults injected with *dsObDll*. We detected no obvious differences in these knockdown phenotypes across both species except for horns, which were affected by *Dll* RNAi in an unexpectedly complex manner. In *O. taurus*, *OtDll* RNAi resulted in a moderate yet significant reduction in head horn length in males normally fated to express a full set of head horns ($t = 10$, df = 22, $P < 0.01$); Fig. 3*Iii*). However, head horn expression in small and medium-sized males was unaffected (Fig. 3*Ii*), as was the expression of pupal thoracic horns in both males and females. In contrast, in the congener *O. binodis*, *ObDll* RNAi resulted in significant reduction in pupal thoracic horn length in both sexes (females: $t = 3.80$, df = 36, $P = 0.0005$, Fig. 3*Iii*; males: $t = 2.08$, df = 34, $P = 0.045$). These results suggest that *Onthophagus Dll* may regulate horn expression in different body regions in a body size- as well as species-specific manner.

Knockdown Validation and Control Injections. We used Western (*dac*, *hth*) and Northern (*Dll*) blot analyses to evaluate the depletion of pupal tissue-specific protein and mRNA levels after RNAi-mediated knockdown (Fig. S3). *dac* RNAi and *hth* RNAi resulted in substantial reductions in gene product across all tissues compared with wild type. Northern blot data are consistent with the hypothesis that *Dll* mRNA is also depleted in a non-tissue-specific manner across tissues as compared with wild type. Combined, these results suggest that dsRNA injections used in the present study degrade their designated targets in a non-tissue-dependent manner.

Control Injections with dsRNA derived from a BlueScript plasmid vector sequence had no obvious phenotypic consequences and did not result in any phenotypes comparable to the RNAi-mediated transcript knockdown experiments described previously. Similarly, control RNA injections did not result in significant changes in horn length-body size allometries in either species (Fig. S4). This observation suggests that injection of dsRNA by itself is not sufficient to cause artefactual pertubations of horn growth and patterning in *Onthophagus* development. This conclusion is further supported by the *dac* RNAi results presented previously, which left horn expres-

sion and allometries similarly unaffected while yielding severe phenotypes elsewhere in the body. Last, neither experimental nor control injections resulted in any obvious changes in developmental timing after injections.

Discussion

The results presented here demonstrate the functional significance of 3 major, traditional appendage patterning genes during the development and diversification of beetle horns. Specifically, larval RNAi-mediated transcription depletion to a level sufficient to cause obvious and often severe effects in other appendages showed that *Onthophagus Dll* and *hth*, but not *dac*, alter horn expression in a species-, sex-, body region-, and body size-specific manner. We briefly discuss the most important implications of our results:

Our results show that even though *Otdac* is widely expressed during prepupal horn primordia (32), it does not appear to play an obvious role in the regulation of size, shape, or identity of horns. Instead, *Otdac* RNAi individuals expressed thoracic and head horns precisely the same size and overall shape as their untreated or sham-treated counterparts despite severe *dac* knockdown phenotypes elsewhere in their body. These results suggest that *dac* expression is not required for horn formation and is either coincidental or vestigial.

In contrast, *hth* transcript depletion had a dramatic effect on horn expression, but only in the thorax. Specifically, *hth* transcript depletion resulted in drastically shortened thoracic horns over the entire range of body sizes but had no effect on head horn expression. Instead, *Othth* RNAi individuals expressed head horns precisely the same size and overall shape as their untreated or sham-treated counterparts despite severe effects elsewhere in the head, including transformations of other head appendages such as the labium and antenna. These results suggest that even though *hth* is expressed during the development of both head and thoracic horns (32), it is required only for the development of the latter. Head horn development, in contrast, appeared remarkably resilient to *hth* transcript depletion. Given the importance of *hth* as a transcriptional cofactor to Hox genes (40, 41), the absence of *hth* transcript depletion-mediated effects on head horn development raises the possibility that head horns, but not thoracic horns, develop without regulatory input from Hox genes. More generally, these results support the hypothesis (11, 14, 32, 33) that different horn types rely on different patterning mechanisms to regulate their expression, and they may have had different and independent evolutionary origins and histories.

Alternatively, the effect of *hth* RNAi on thoracic horn development could have been secondary and reflective of *hth*'s role in establishing the identity of the prothoracic segment. For example, the expression of ectopic wing tissue on both sides of the pronotum in response to *hth* RNAi may be diverting resources away from horn growth, and a reduction in thoracic horn size may then be a correlated response to *hth* RNAi rather than reflective of a true patterning function during thoracic horn development. If correct this possibility would suggest that the expression of *hth* at the base of developing thoracic horns reported earlier (32) may be coincidental and not reflective of proximal horn patterning. We are presently not in a position to reject this alternative explanation with the data at hand.

Examination of *Onthophagus Dll* function further complicated our understanding of developmental evolution of beetle horns. Unlike *hth*, *Dll* transcript depletion affected the expression of both head and thoracic horns. However, in *O. taurus* head horn expression was affected only in large males otherwise fated to express a full set of head horns, whereas horn expression in small and medium-sized males was unaffected, as was the expression of pupal thoracic horns in both males and females regardless of body size. In contrast, *Dll* RNAi affected thoracic horn expression in the congener *O. binodis*, and did so in both males and females, although the effect was strongest in large individuals. These results suggest that *Onthophagus Dll* regulates horn expression in a body region- and body size-dependent manner, and that even closely related species can diverge rather substantially in the degree to which development of the same horn type is affected by the same gene.

Apart from horn development, however, larval RNAi-mediated transcript depletion of all 3 patterning genes generated phenotypic effects very similar to those documented in previous studies, including loss and fusion of appendage regions, alterations of eye development, homeotic transformations, and expression of ectopic wing tissue. Combined, our results therefore suggest that horn development evolved via co-option of at least some p/d-axis patterning genes, and that this co-option was feasible without compromising the ancestral function of these patterning genes in the development of already established traits.

On one hand, our results are thus not surprising and confirm a general pattern in the evolution of novel traits: new morphologies do not require new genes or developmental pathways and instead may arise by recruiting existing developmental mechanisms into new contexts (5–7). On the other, our results revealed an unexpected degree of evolutionary lability, ranging from the absence of patterning function (*dac*) to patterning function in selected horn types only (*hth, Dll*) to function in 1 size class, sex, or species but not another (*Dll*). Combined, these results contradict the notion that upstream regulators, such as p/d-axis patterning genes, should be evolutionarily entrenched and conserved given their importance in the regulation of basic aspects of animal architecture (42, 43). Instead, our results illustrate that regulatory genes whose functions are otherwise highly conserved nevertheless retain the capacity to acquire new functions, and that little phylogenetic distance is necessary for the evolution of sex- and species-specific differences in these functions. We speculate that such differential recruitment of patterning genes and subsequent diversification of patterning function are likely to have occurred numerous times during the evolutionary history of beetle horns as different horn types have likely originated independently of one another and development of similar horn types most likely arose several times independently in and outside the genus *Onthophagus* (11, 20, 33).

Methods

Animal Husbandry. *O. taurus* and *O. binodis* were collected in the field and reared in the laboratory as described in ref. 32. Larvae were sexed as described in ref. 44.

Cloning. *Otdac* was cloned as described in ref 32. *Obhth* was cloned using PCR Taq DNA Polymerase (Bioline) with sense (5′-TTYAAYGARGAYATHGCNRT-3′) and antisense (5′-CARGCIATMCARGTICTBMGGTT-3′) primers and nested sense (5′-ARRTCDATNGGCATYTTNCCYTT-3′) and antisense (5′-CARGCIATMCARGTICTBMGGTT-3′) primers and *O. binodis* cDNA as template. PCR products were purified and subcloned into pCRII-TOPO vector by using the TOPO TA Cloning kit (Invitrogen). *ObDll* was cloned by using PCR Taq DNA polymerase with sense (5′-TAYCCYTTCSSKCCCATGCAC-3′) and antisense (5′-TGMGCMGCCTTCATCATCTTYTT-3′) primers and nested sense (5′-TAYCCYTTCSSKCCCATGCAC-3′) and antisense (5′-GGNGGNAARGGNAARAAATGMG-3′) primers and *O. binodis* cDNA as template. *OtDll* was cloned by using PCR Taq DNA polymerase with sense (5′-CAYGARTCNAAYACNTCNACNCC-3′) and antisense (5′-GGNGGRCARTCNGCNGCRTA-3′) primers and nested sense (5′-TTYATYGARYTNCARCARCA-3′) and antisense (5′-GGNGGRCARTCNGCNGCRTA-3′) primers and *O. taurus* cDNA as template. All *Dll* PCR products were purified and subcloned into the pSC-A vector by using the Strataclone PCR Cloning kit (Stratagene). All constructs used were sequenced by using the BigDye Terminator Sequencing kit (Applied Biosystems). Sequence reads were prepared on an Applied Biosystems ABI Prism 3730 sequencer.

dsRNA Construction. In vitro transcription using T7 and SP6 RNA polymerase (*OtDll, ObDll, Othth*) or T7 and T3 RNA polymerase (*Otdac*) was carried out as specified by the manufacturer (MEGAscript kit, Ambion) to produce both sense and antisense RNA strands for each of the fragments. Equimolar amounts of complementary strands were mixed and samples were heated to 95 °C for 3 min then slowly cooled over 4 h to 25 °C.

RNAi Injection. Larvae were injected up to 10 days after molt to the third instar (≈5 days before the gut purge). Three microliters of a solution containing

0.5–5 μg of dsRNA in injection buffer (5 mM KCl, 1 mM KPO_4 pH 6.9) was loaded into a gas-tight 1801 Hamilton syringe with a 32-gauge needle (Hamilton) and injected medial just behind the metanotum. After injection, larvae were allowed to develop individually in transfer plates until scoring at pupal and day 2 adult timepoints. Control animals consisted of (*i*) untreated animals reared under the same conditions and (*ii*) animals injected in parallel to RNAi individuals with a 167-bp portion of Bluescript SK+ vector in injection buffer.

Allometric Measurements and Analysis. RNAi treated and control pupae and adults were measured by using a 2D image analysis setup consisting of a dissecting microscope (Leica) mounted with a digital camera (Scion) and ImageJ software. Thorax width and body mass were used as measures of pupal and adult size. Thoracic and head horn length were measured as described in refs. 13 and 15. Measurements were recorded to the nearest 0.001 mm.

Statistical Analysis. Allometric scaling relationships of control and RNAi-treated individuals were analyzed in line with previous studies (e.g., refs. 33 and 45–47) by using a residual analysis as detailed in ref. 48. We used two-tailed *T* tests to determine whether horn length residuals differed significantly across treatment groups.

Western and Northern Analysis. We used Western (*dac, hth*) and Northern (*Dll*) analyses to validate gene product depletion in a tissue-specific manner. Details of the analyses are described in *SI Methods*.

Sequence Analysis. Putative *hth* and *Dll* sequences were identified by basic local alignment search tool (BLAST) analysis. Multiple alignments of protein sequences were constructed by using the ClustalW program and the alignments were plotted in Boxshade (Figs. S1 and S2). Nucleotide sequences were aligned using ClustalW and evolutionary relationships were inferred using the neighbor-joining algorithm in the MEGA 4.0 software package with bootstrapping (1,000 iterations). Evolutionary distances were computed by using maximum composite likelihood. Rate variation among sites was modeled with gamma distribution and all positions with gaps were treated by the complete-deletion method (49). To explore differences in topology that might be clarified by a more complex model, trees were also constructed by using the maximum likelihood (ML) method, a heuristic tree search, using PAUP version 4.0b10. The best model for both genes was GTR+I+G. A TBR branch-swapping algorithm was used (50). The two methods yielded similar topologies.

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