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Programed cell death shapes the expression of horns within and between species of horned beetles

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SUMMARY Holometabolous insects provide an excellent opportunity to study both the properties of development as well as their evolution and diversification across taxa. Here we investigate the developmental basis and evolutionary diversification of secondary trait loss during development in the expression of beetle horns, a novel and highly diverse class of secondary sexual traits. In many species, horn growth during late larval development is followed by a period of dramatic remodeling during the pupal stage, including the complete resorption of horns in many cases. Here we show that programed cell death plays an important and dynamic role in the secondary resorption of pupal horn primordia during pupal development. Surprisingly, the degree of cell death mediated horn resorption depended on species, sex, and body region, suggesting the existence of regulatory mechanisms that can diversify quickly over short phylogenetic distances. More generally, our results illustrate that secondary, differential loss of structures during development can be a powerful mechanism for generating considerable morphological diversity both within and between species.

INTRODUCTION

A basic objective of developmental biology is to elucidate the mechanisms by which traits originate and change during an organism's ontogeny. A basic objective of evolutionary developmental biology is to understand how novel traits arise in the first place, and how ontogenies themselves change over evolutionary time scales (Gilbert and Epel 2009). Insects undergoing complete, or holometabolous, metamorphosis, provide an excellent opportunity to study both phenomena in the same organisms: on one side, individual ontogenies are marked by dramatic changes in size, shape, and organization as individuals molt from larvae to pupae and adults. On the other, holometabolous development is itself highly diverse, and many taxa may appear similar at some life stages yet express very different phenotypes, including novel traits, at others.

In many holometabolous insects, the pupal stage often gives the first strong indications of future adult morphology. For instance, wings, legs, antennae, and general body organization of many adult Lepidoptera (butterflies and moths), Coleoptera (beetles), Hymenoptera (ants, bees, wasps, sawflies), and other holometabolous insect orders are already visible and distinguishable in the corresponding pupae (Grimaldi and Engel 2005). In these cases differences among adults are the product of differential growth of parts before the larval–pupal molt, causing adults of different taxa to exhibit the same basic phenotypic difference as were already evident during the preceding pupal stage. In other cases, prepupal growth and development may be partly or fully similar in different individuals or taxa, yet followed by secondary loss of traits during subsequent development in at least some individuals. In holometabolous insects this secondary loss of traits appears to play a particularly important role in the generation of caste and sexual dimorphisms. For instance, wingless workers of some ant species in the genus *Pheidole* undergo normal wing development until the prepupal stage, during which wing primordia are then resorbed by apoptosis (Sameshima et al. 2004). Secondary loss of wings is even more extreme in the tussock moth, *Orgyia recens* (Lobbia et al. 2003; Lobbia et al. 2007). Here, male and female wing development is indistinguishable until early pupal development and results in complete and similarly sized pupal wings in both sexes. However, following pupation only male pupal wings become transformed to adult structures whereas female wings are almost completely resorbed via programed cell death (PCD), resulting in highly vestigial wings in female moths only. Importantly, phenotypic differences among the resulting adults leave few if any clues regarding the nature and timing of the underlying developmental mechanisms. Here, we investigate both the developmental basis and evolutionary diversification of secondary trait loss during development in the expression of beetle horns, a novel and highly diverse class of secondary sexual traits.

Many species of beetles express prominent horns or horn-like structures, cuticular projections of the head and/or prothorax. Horns lack obvious homology to other insect structures and therefore constitute an evolutionary novelty

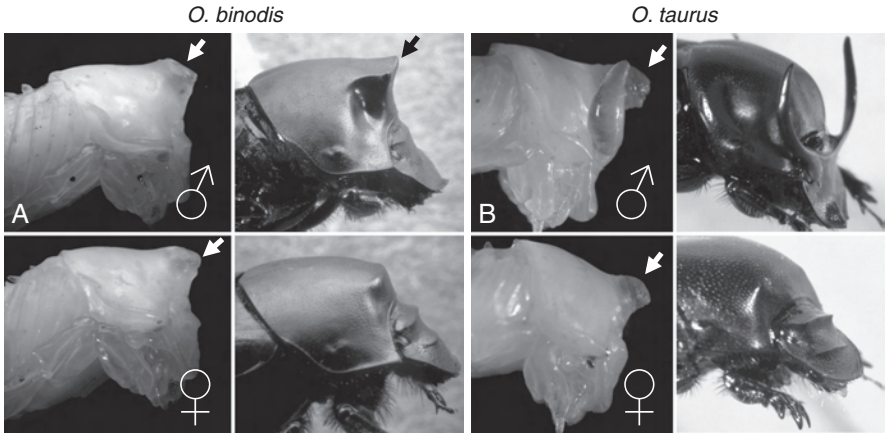


Fig. 1. Adult and pupal horn morphology of the beetle species used in this study. *Onthophagus binodis* (A) and *Onthophagus taurus* (B) with male (upper row) and female (bottom row) are shown. Left column of each species represents pupae. Note that horn resorption occurs only in female of *O. binodis* but in both sexes of *O. taurus*. Arrows indicate prothoracic horns. Each image shows the lateral prothorax and head.

that, since its invention, has undergone tremendous diversification both within and between species (reviewed in Emlen et al. 2007, Snell-Rood and Moczek in press). Horns function as weapons in male competition over females, and in almost all species horn expression is either confined to, or greatly exaggerated in, males. In addition, species differ widely in location of horn expression, horn size and shape, as well as number of horns expressed by individuals. Horns are most frequent and diverse within the family Scarabaeidae (Arrow 1951). Here the highly diverse genus *Onthophagus* offers the opportunity to study the development and diversification of horns within a narrow phylogenetic framework. Many *Onthophagus* species differ in aspects of horn expression due to differences in prepupal horn growth, causing pupae to exhibit the same morphological differences evident in the resulting adults. However, recent studies have highlighted that in many species prepupal growth of horns, in particular those extending from the thorax, is rather uniform. In these cases, many sex- and species-specific differences in horn expression appear to arise from differential resorption of horn tissue during the pupal stage, oftentimes allowing fully horned pupae to molt into entirely hornless adults (Moczek et al. 2006). The underlying developmental mechanisms, however, are unknown. Here, we show that PCD is responsible for the resorption of pupal horns in a species, sex, and body-region specific manner, and therefore constitutes an important development mechanism underlying morphological diversity in *Onthophagus* beetles.

MATERIALS AND METHODS

Species choice

We investigated the developmental and cellular mechanisms underlying pupal remodeling of horn primordia in *Onthophagus binodis* and *Onthophagus taurus*. In *O. binodis*, both males and females grow a similarly sized prothoracic horn during prepupal development, which becomes visible externally during the pupal stage (Fig. 1). However, only male *O. binodis* convert the pupal prothoracic horn to an adult structure, whereas females largely resorb it before the pupal-to-adult molt (Fig. 1A). In contrast, *O. taurus* expresses two types of horns: both males and females express a medial prothoracic horn, and large males only also express a pair of large curved head horns. Both horn types are grown during the prepupal stage and externally visible in pupae, but only head horns are carried through to the adult stage whereas the medial prothoracic horn is resorbed entirely in both sexes (Fig. 1B).

Species husbandry

Laboratory colonies of both species were derived from field populations. *O. taurus* was collected from pastures around Bloomington, IN, and *O. binodis* was collected from pastures near Waimea, Hawaii. Both species were maintained and reared as described previously (Moczek 2006). Early third instar larvae of each species were transferred from their natural brood ball into 12-well plates to monitor larval development daily. First to second-day pupae were then sexed and treated as described below. We detected no difference in the duration of the pupal stage between the sexes (approximately 12 days for *O. binodis* and 9 days for *O. taurus*).

Fixation of samples and preparation of cryosections

Pupae collected within 24 h after pupation are referred to as day 1 pupae, whereas pupae collected within 48 h are referred to as day 2 pupae in this study. Samples were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffered saline (2% PFA/PBS). Approximately 100 µl of fixative were first injected from the ventral side of second thoracic segment, followed by an additional injection of approximately 400 µl into the dorsal abdomen. *O. binodis* samples were soaked in 2% PFA/PBS for 2 h to overnight at 4°C. After fixation, samples were rinsed with PBS for 15 min at room temperature (RT) five times followed by soaking in a series of decreasing ethanol concentrations (80%, 60% with water, and 40% with PBS) for 20 min at 4°C. The samples were then rinsed with PBS three times at RT followed by soaking in 30% sucrose overnight at 4°C. The equilibrated samples were embedded in O.C.T. compound (IMEB, San Marcos, CA, USA), frozen, and stored at − 80°C. Frozen samples were sectioned using a cryostat (Microm; Heidelberg, Germany). Twenty micrometer

thick sections were prepared and placed on microscope slides (VWR, West Chester, PA, USA) and stored at −20°C until further use.

O. taurus samples were treated identical to *O. binodis* samples with the following exceptions. *O. taurus* heads were dissected after injection of 2% PFA/PBS, followed by further fixation of samples in 2% PFA/PBS overnight at 4°C. In addition, we omitted ethanol treatment steps for these samples.

Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) and immunodetection

We used the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA) to detect cells undergoing programed cell death on pupal sections. Basic procedures were performed according to the manufacturer's instructions except for incubation time during "permeabilization" (which was increased from 2 to 7 min on ice) and TUNEL reaction (which was increased from 1 to 2 h at 37°C). Then sections were rinsed in PBS three times at RT to terminate TUNEL reaction. Immunodetection of putative *Onthophagus* DRICE homolog was then performed by using rabbit antiserum against active *Drosophila* effecter caspase (DRICE, Yoo et al. 2002; antibody courtesy of Bruce Hay). Samples were incubated with 5% normal goat serum in PBS (blocking solution) overnight at 4°C. Rabbit anti-active DRICE antiserum was diluted 1 to 250–750 in the blocking solution and the samples were incubated overnight at 4°C. After rinsing with PBS five times for 10 min at RT, secondary antibody solution (1/250 diluted goat anti-rabbit antibody labeled with Cy3 in blocking solution) and DAPI (1/1000) was applied for 1 h at RT. Then we rinsed the samples with PBS three times for 10 min at RT followed by rinsing with water immediately before mounting in Aqua Poly/Mount (Polysciences, Warrington, PA, USA). We used a Nikon800 (Nikon, Tokyo, Japan) Fluorescent Compound Microscope and MetaMorph imaging software (Molecular Devices, Sunnyvale, CA, USA) to obtain and analyze images. Adobe Photoshop CS4 was used to process images.

Filtering of candidate genes for the regulation of PCD from microarray results

Results from a companion study (Kijimoto et al. 2009) allowed us to identify a gene list of putative cell death-related genes that may function during horn resorption. Specifically, Kijimoto et al. 2009 used day 1 pupae of male *O. taurus* to contrast relative gene expression levels between head horns, prothoracic horns, and legs to those detected in abdominal epithelium. In the present study we reanalyzed the gene list generated by Kijimoto et al. 2009 and manually filtered those genes whose homologous protein names from UniProtKB or FlyBase, or whose Gene Ontology terms (Ashburner et al. 2000, FlyBase), contained one or more of the following terms: death, apoptosis, apoptotic, autophagy, autophagic. This filtering procedure identified 59 array spots, 28 of which indicated significant enrichment or depletion of the corresponding expressed sequence tag (EST) relative to expression levels in the abdominal epithelium. Lastly, we excluded those ESTs whose differential expression was restricted only to legs. This resulted in a list of 14 putative cell death-related genes that were differentially enriched or depleted in prothoracic horns, head horns, or both.

RESULTS

Recall that pupal prothoracic horns are resorbed only in female *O. binodis*, but in both sexes in *O. taurus* (Fig. 1). We hypothesized that PCD may mediate sex- and species-specific resorption of the prothoracic epithelial cell layer. Thus, we used two bioassays to detect common PCD markers. Specifically, we used TUNEL to detect genomic DNA fragmentation and antiserum against active *Drosophila* effector caspase, which in its activated form functions in protein degradation during PCD.

In *O. binodis*, only females showed obvious epithelial cell layer detachment during the time period between 24 and 48 h (day 2) after pupation, followed by nearly complete resorption of the pupal prothoracic horn (Fig. 2, A–D). In contrast, male *O. binodis* did not show signs of early epithelial cell layer detachment and instead retained their pupal prothoracic horn into adulthood (Fig. 2, N and O). In *O. binodis* both male and female pupae less than 24 h old (day 1) showed very little, if any, signs of PCD in the pupal prothoracic horn (female: Fig. 2, A and B; male not shown). However, sexes began to differ in the occurrence of PCD during the second day of pupal life (Fig. 2, C and D, and I–T). Day 2 females showed considerable enrichment of PCD in detached epithelium (Fig. 2, I and J). Specifically, we observed cytosolic localization of putative active caspase (Fig. 2L) and TUNEL positive cells (Fig. 2M). Furthermore, the morphology of the epithelial cell layer underlying the pupal prothoracic horns appeared to change between days 1 and 2. During this time period the epithelium increased in thickness and included many condensed nuclei, another characteristic feature of PCD (Fig. 2K). Because of at times significant background of putative anti-active caspase staining we were not able to fully distinguish between background staining and moderate expression (e.g., Fig 2G). However, we observed that during day 2 TUNEL signal consistently co-occurred with caspase signal, and was commonly accompanied by condensation of cell nuclei. The combination of epithelial detachment, nuclear condensation, and co-elevated TUNEL and anti-caspase staining observed during day 2 suggest that PCD during female prothoracic horn development is likely most prevalent at this stage of pupal life.

In contrast, day 2 male *O. binodis* pupae showed only minor enrichment of PCD signals (Fig. 2, P–T) compared with day 2 females. While we did detect a few interspersed TUNEL-positive cells in the day 2 male pupal prothoracic epithelium (arrows in Fig. 2, Q–T), we failed to detect the localized extensive PCD signals characteristic of the female pupal prothoracic epithelium at the same developmental stage. These results are consistent with the hypothesis that sex-specific PCD occurring in the prothoracic horns of day 2 female, but not male, pupae contributes to the sex-specific resorption of the prothoracic horn of female, but not male, *O. binodis*.

We also detected TUNEL signal in other tissues such as legs, mouthparts, and dorsal abdominal segments, regardless

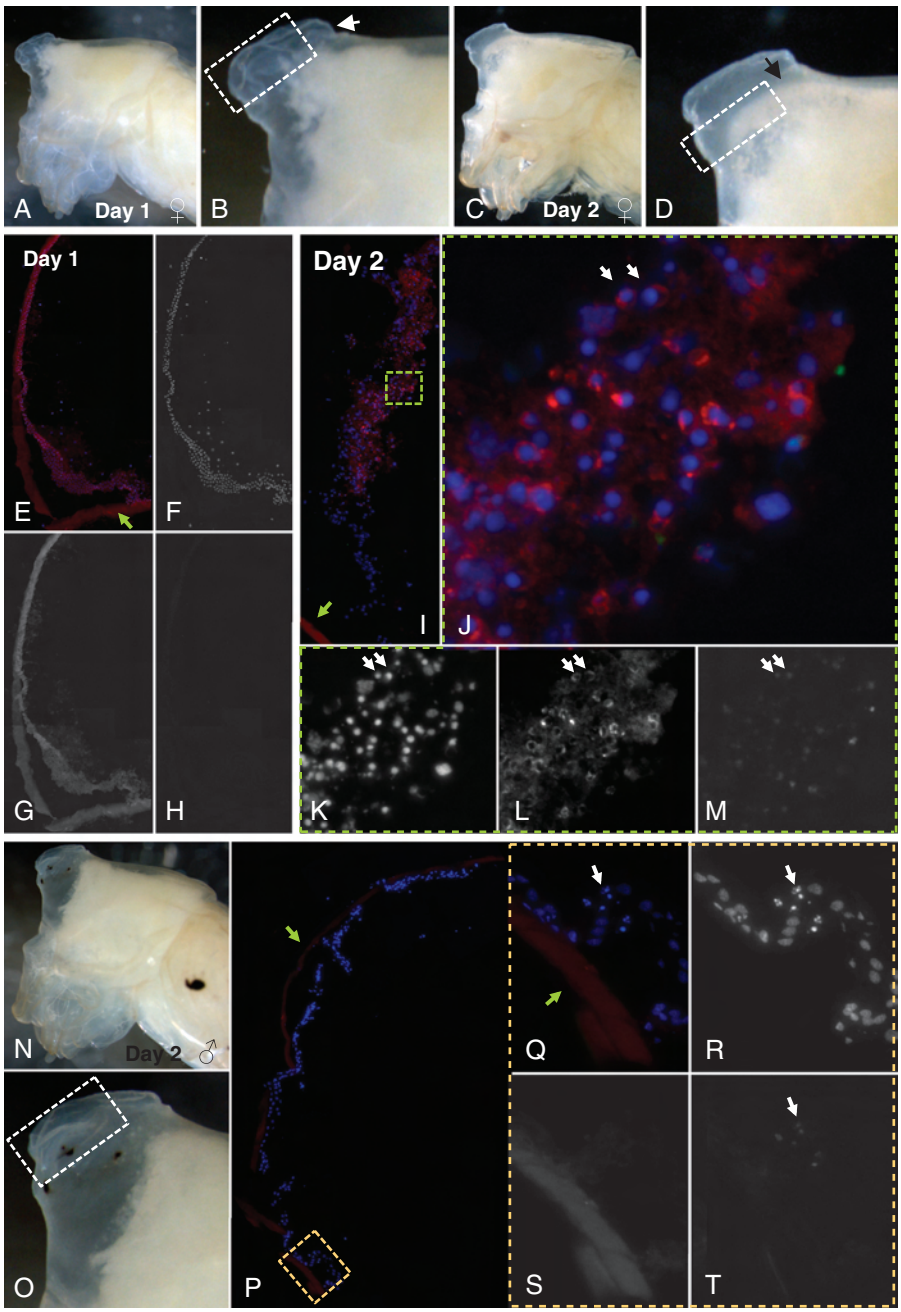


Fig. 2. Programed cell death (PCD) is detected during the second day of pupal life in female *O. binodis* pupae (A–M) but only to a small degree in prothoracic horns of corresponding male pupae (N and O). Lateral view of (A) day 1 female pupa with (B) magnified view of prothoracic horn and (C) day 2 female pupa with (D) magnified view (anterior is to the left). In early day 1, the epithelium of the prothoracic horn has not yet detached (arrow in B), while the epithelial cells detach from the cuticle in day 2 (C and D). Dotted boxes in (B) and (D) are approximately the same size as images (E) and (I), respectively. During day 1, little PCD signal is detected (E, merged image of blue: DAPI staining; orange: anti-caspase staining; green: terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling [TUNEL]). Except for possible background of anti-caspase antibody (G), (F) DAPI and (H) TUNEL images indicate little signs of PCD. In contrast, during day 2 (I), detached epithelium shows extensive PCD signal. (J) Magnified view of the green dotted box indicated in (I) with (K) DAPI, (L) anti-caspase; and (M) TUNEL. White arrows indicate examples of cells visible in panels J–M. (N and O) Day 2 male pupae of *O. binodis* do not show significant detachment of epithelial cells from the cuticle as well as little PCD signal (P). (Q) Magnified views of the yellow dotted box in (P) indicate (R, DAPI) a few condensed nuclei, (S) little caspase expression, and (T) little TUNEL signal (compare with J–M). White arrows indicate an example of a cell visible in panels Q–T. Note that cuticle tends to attract the antibody, resulting in relatively strong nonspecific staining (green arrows in E, I, P, and Q).

of sex (data not shown). These results show that PCD itself takes place broadly during the pupal stage, but that sex-specific increases of PCD appear restricted to the female prothoracic horn.

Results for the congener *O. taurus* were only partly similar. In this species both males and females resorb their prothoracic horns during the pupal stage (Fig 1B). In addition, male *O. taurus* develops a pair of head horns, which undergo minimal if any, resorption. We therefore explored both horn types separately, focusing first on the prothoracic horn.

In *O. taurus*, both males as well as females showed significant detachment of the prothoracic epithelial cell layer from the pupal prothoracic cuticle, followed by complete resorption of the pupal prothoracic horn in both sexes (male:Fig. 3, A–D; female not shown). As with *O. binodis*, both male and female pupae in day 1 showed little if any, obvious signs of PCD in the pupal prothoracic horn (male: Fig. 3, A and B, and E–I; female not shown) except for moderate anti-caspase staining (e.g., Fig. 3H). However, all other observations suggest that—similar to *O. binodis*—PCD

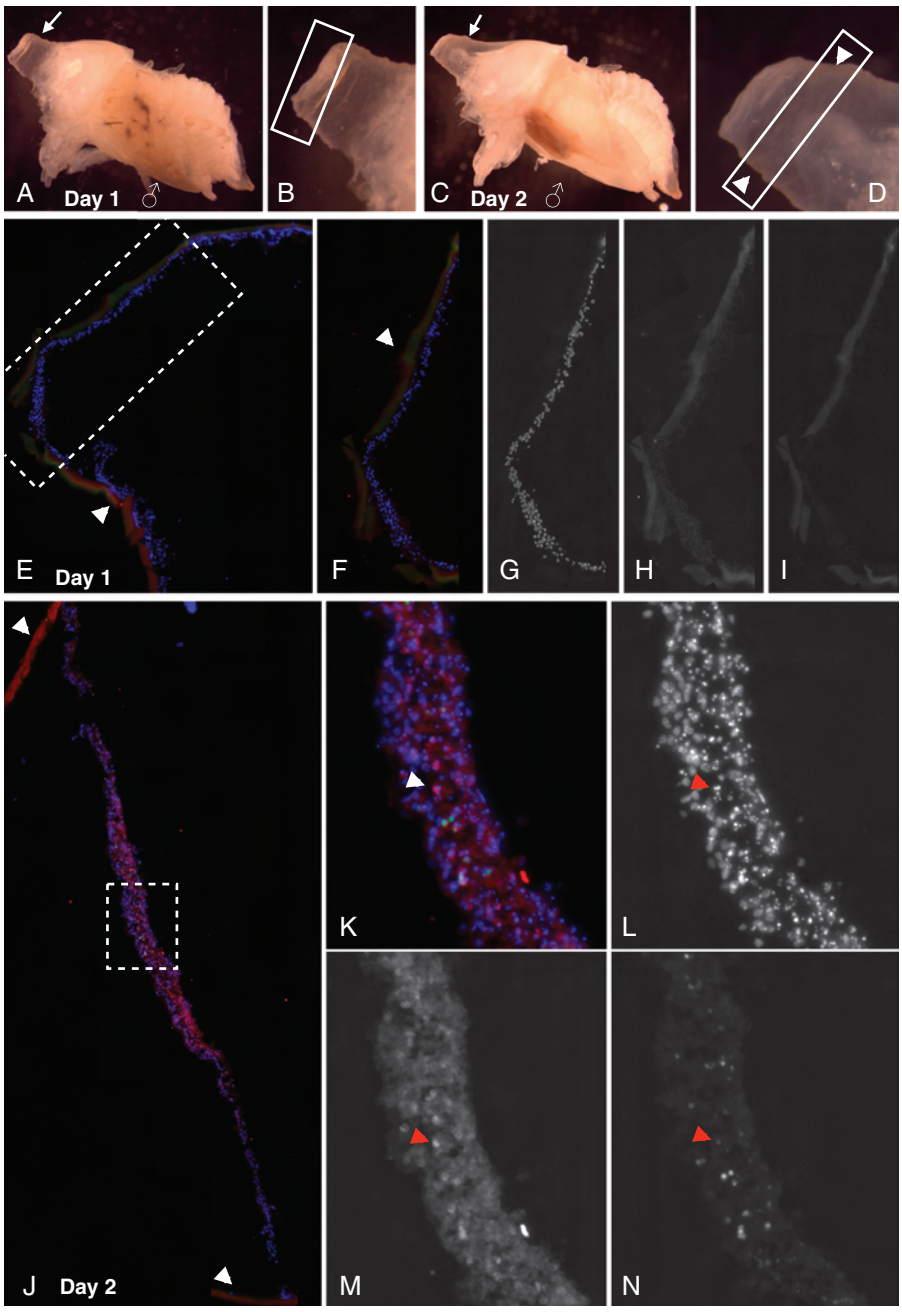
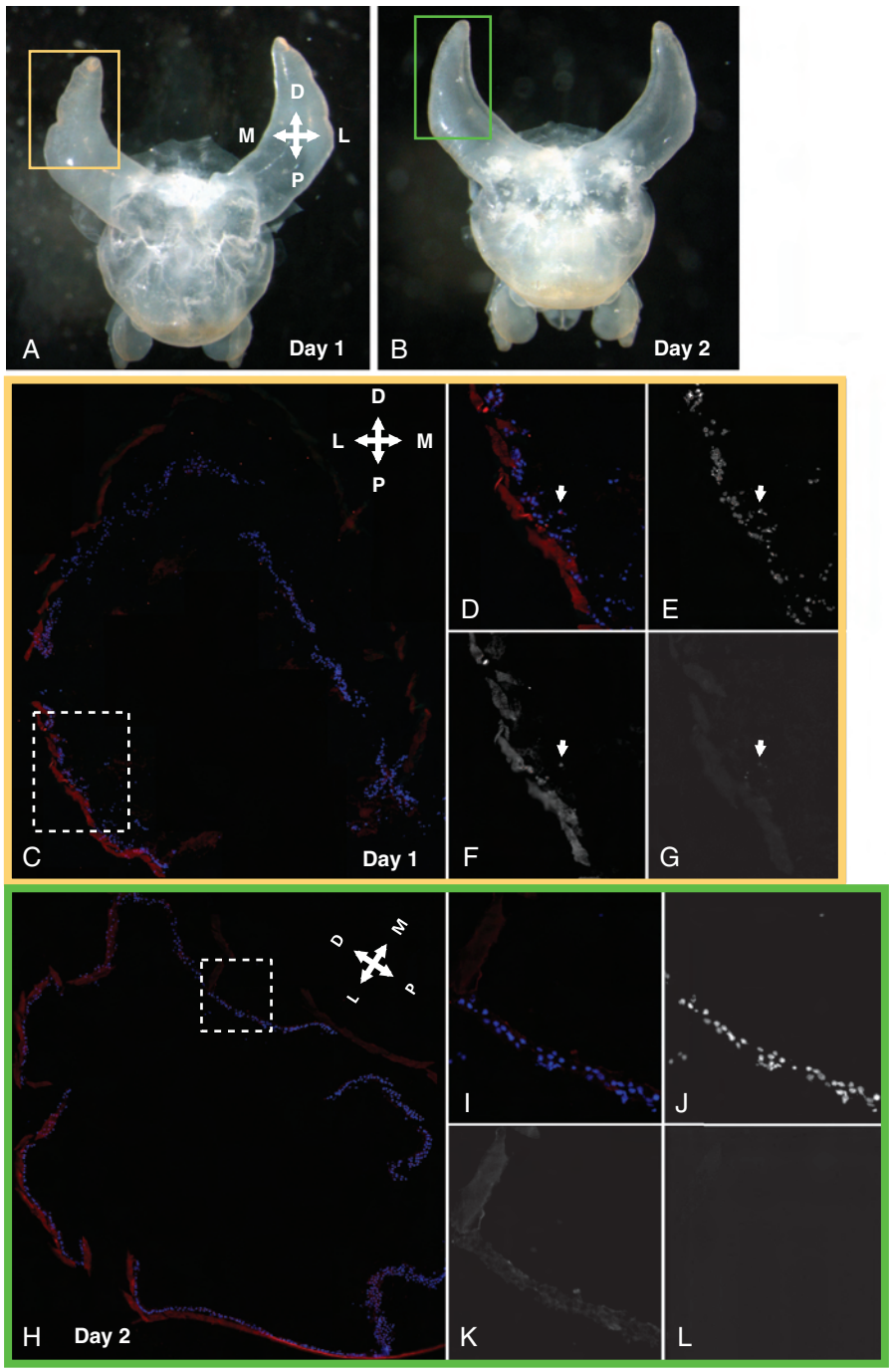


Fig. 3. Programed cell death (PCD) is detected in the prothoracic horns of day 2 *O. taurus* male pupae. (A–D) Lateral view of (A) day 1 male with (B) magnified view and (C) day 2 male with (D) magnified view (anterior is to the left). Note that heads are removed from the samples to better observe prothoracic horns. Detachment of the prothoracic epithelium from the cuticle does not occur until day 2 of pupal life (indicated by arrows in A and C). The boxes in (B) and (D) indicate the area examined in more detail in (E) and (J), respectively. (E) During day 1, little PCD signal is detected in prothoracic horns. (F–I) magnified view of dashed box in (E). (G) DAPI; (H) anti-caspase antibody staining; (I) terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL). (J) Day 2 male prothoracic horn epithelial cells show localized tissue thickening as well as PCD signals. Arrowheads indicate background staining of cuticle (compare with arrowheads in D). (K–N) Magnified views of dashed box indicated in (J) show that PCD signals appear enriched compared with day 1 (K: merged image; L: DAPI, M: anti-caspase, and N: TUNEL). Arrowheads in K–N indicate an example of a cell indicating PCD. Arrowheads in (E), (F), and (J) indicate nonspecific anti-caspase staining in the cuticle. Arrowheads in (J) indicate pupal cuticle also highlighted in (D).

in the prothoracic pupal horns of *O. taurus* may occur primarily during the second day of pupal life. Specifically, the combination of abundant TUNEL-positive cells, strong, localized signal of putative anti-active caspase staining, and condensed nuclei in thickened epithelial cell layers was observed first in day 2 pupal prothoracic epithelium (Fig. 3, J–N). Unlike in *O. binodis*, in *O. taurus* both sexes exhibited these extensive PCD signals in the pupal prothoracic epithelium, from detachment and thickening of the prothoracic epithelial cell layer to considerable TUNEL signal, anti-caspase staining, and condensation of nuclei (Fig. 3, C and D, J–N, female not shown). More generally, these results indicate that in *O. taurus* PCD in the pupal prothoracic horn is not restricted to females, unlike in *O. binodis*, but instead occurs in both sexes, consistent with the resorption of prothoracic horns seen in both male and female *O. taurus*.

Despite the substantial PCD signal detected in prothoracic horns, male head horns of *O. taurus* failed to reveal obvious signs of PCD in both day 1 and 2 pupae (Fig. 4). Even though epithelial cells underwent slight detachment from the cuticle during day 2 (compare Fig. 4, A and B), we detected substantially less PCD signal than in prothoracic horns (compare



Colour

Fig. 4. Only minimal programed cell death (PCD) is detected in the head horns of day 2 *O. taurus* male pupae. Dissected heads of (A) day 1 and (B) day 2 *O. taurus* male pupae (frontal view) as well as corresponding sections of head horns from each stage (C–G and H–L, respectively). The yellow box in A and the green box in B correspond to the same areas shown in C and H, respectively. In contrast to the significant amount of PCD seen in prothoracic horns of the same *O. taurus* male individuals (Fig. 3) as well as *O. binodis* females (Fig. 2), only a few PCD signals could be observed in head horns of male *O. taurus* pupae. (C) On day 1, only the region boxed by white dashes (D) showed PCD signal (E: DAPI; F: anti-caspase; G: Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling [TUNEL]). (H) On day 2, the region boxed by white dashes (I) showed relatively stronger signal of anti-caspase staining (K), but DAPI (J) and TUNEL (L) signals were inconclusive. Proximal–distal (P–D) and lateral–medial (L–M) axes of the horns are shown.

Fig. 4, C–G with Fig. 4, H–L). This result suggests that PCD-mediated horn resorption occurs in a horn type-specific manner in male *O. taurus*.

Reanalysis of a recent companion study designed to contrast transcription profiles of prothoracic horns, head horns and legs in *O. taurus* (Kijimoto et al. 2009) allowed us to identify genes that may function in PCD and/or its regulation as differentially expressed in developing horns. The original analysis compared gene expression levels between prothoracic horns, head horns, or legs with that of abdominal epithelium of the same individuals using day 1 pupae. Our results presented above suggest that the *execution* of PCD in horns does not occur until day 2. However, genes involved in the differential *regulation* of PCD may already be differentially expressed in day 1 animals, and thus may be detectable by our approach. 59 spots on the microarray met our criteria, and

14 genes were found to be significantly enriched or depleted in prothoracic horns (see "Materials and Methods" for the filtering criteria). Among the 14 candidate genes, five were significantly enriched in prothoracic horns (Table 1), including the transcription factors *broad* and *E93*, which play important roles in ecdysteroid induced regulation of PCD during *Drosophila* development. A subsequent search for genes related to ecdysteroid biosynthesis identified a sixth gene, *shade*, to be significantly enriched in prothoracic horns (Table 1). Associated GO terms did not explicitly link *shade* to PCD, however, *shade* function is critical for converting ecdysone to its more active form, 20-hydroxyecdysone (20E, reviewed in Gilbert and Warren 2005), which in turn is likely to regulate the expression of *broad* and *E93* (Wu et al. 2006). These results thus provide additional evidence that important components of the PCD machinery in insects are likely to have been recruited into the development of beetle horns, and that evolutionary changes in the regulation of PCD during horn formation have contributed to the diversification of horned beetles.

DISCUSSION

In this study, we showed that PCD is associated with the sex- and species-specific resorption of pupal prothoracic, but not head, horns of *Onthophagus* beetles. Specifically, we showed that each instance of pupal prothoracic horn resorption (female *O. binodis*, male and female *O. taurus*) was marked by detachment of the pupal epithelium and substantial PCD during early pupal development. In contrast, no early detachment and elevated PCD was detected in instances in which prothoracic horns were maintained into adulthood (male *O. binodis*). These results support the hypothesis that sex-specific modulation of PCD mediates the expression of sexual dimorphism in *O. binodis*. Moreover, these results show that closely related species can diverge with respect to which sex is affected. Lastly, head horn development in male *O. taurus* was marked by little PCD during the same developmental period, suggesting that different horn types, including those expressed by the same individual, are differentially affected by PCD. Below we discuss the most important implications of our findings.

PCD as a system to remove pupal prothoracic horns

Our histological data implicate PCD in the removal of pupal horn primordia. A recent companion study designed to contrast transcription profiles of prothoracic horns, head horns and legs in *O. taurus* (Kijimoto et al. 2009) further supports this hypothesis and begins to suggest possible developmental

Table 1. Summary of possible programed cell death (PCD)-related genes detected thus far in *Onthophagus*

	Microarray results				
ID	Head horns	Thoracic horns	Legs	FlyBase gene description	References
OtL006-A09	2.2	1.9	—	Epithelial membrane protein	Gorski et al. (2003)
OtL001-A07	2.0	1.4	1.3	Broad	Cakouros et al. (2002)
OtL015-H02	1.4	1.4	1.6	CG13393	FlyBase
OtP006-G11	− 1.4	− 1.4	− 1.7	Rab-protein 7	Gorski et al. (2003)
OtL002-A04	− 1.8	− 2.4	− 1.8	PDCD-5	NCBI HomoloGene:10506
OtL013-G08	− 2.0	− 1.9	− 2.0	Cysteine proteinase-1	Gorski et al. (2003)
OtL016-A06	− 2.7	− 2.6	− 3.0	cathD	Gorski et al. (2003)
OtL002-A12	− 6.1	− 12.1	− 19.7	Sclp	Bowler et al. (2006)
OtL017-B11	—	2.3	—	Shade	NA
OtL002-A09	—	1.4	—	eIF-5A	Gorski et al. (2003)
OtP010-C07	—	1.4	—	Eip93F	Lee and Baehrecke (2001)
OtP017-E07	—	− 1.3	—	Darkener of apricot	Gorski et al. (2003)
OtL011-E05	—	− 1.5	—	Spinster	Nakano et al. (2001)
OtL012-D04	—	− 1.6	—	La-related protein	Gorski et al. (2003)
OtL010-F03	—	− 1.6	—	CG7188	FlyBase

Candidate genes were identified by filtering microarray data obtained in a companion study (Kijimoto et al. 2009) as described in "Materials and Methods." Note that transcription profiles in Kijimoto et al. (2009) were quantified using day 1 pupae. Results of the present study suggest that cells undergoing PCD do not become detectable in large numbers until day 2. The array data used here may therefore not be ideal for identifying genes involved in the *execution* of PCD. However, the same data may be useful for identifying genes involved in the *regulation* of differential PCD activation that are already differentially expressed during day 1. Genes in boldface were found to be enriched in prothoracic horns of day 1 male *O. taurus* pupae. Values in microarray results columns indicate the fold differences when compared with abdominal epithelium. Negative values indicate genes that are less expressed (or depleted) in horns compared with abdominal epithelium. "—" values were not obtained because differences across body regions were not significant or signal strength was below background. Also shown are gene descriptions and references to possible functions in the regulation of PCD.

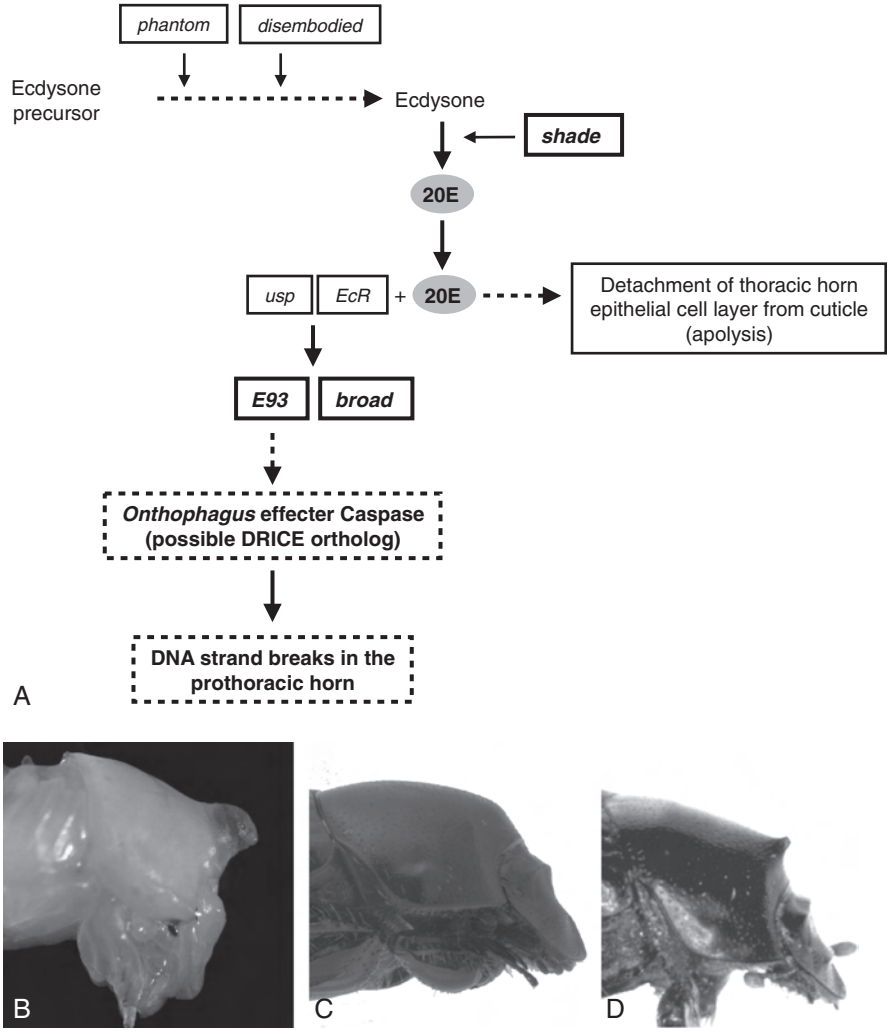


Fig. 5. Programed cell death (PCD) might play a role in resorption of prothoracic horn in *Onthophagus*. (A) Possible model of the regulation of PCD-mediated prothoracic horn resorption. Different types of evidence presented here implicate several components of PCD and its regulation as it is known from other insects in the resorption of pupal horns in *Onthophagus* beetles. This includes the ecdysone biosynthesis pathway (including the genes *phantom* [*phm*] and *disembodied* [*dib*]), ecdysone activation (facilitated by *shade*) and reception (via *ultraspiracle* [*usp*] and *Ecdysone receptor* [*EcR*]), induction of downstream regulators of PCD (including *E93* [*Eip93F*] and *broad*), and caspase-mediated protein digestion and DNA strand breaks. Specifically, genes boxed in bold were found to be enriched in prothoracic horns as well as other appendages using microarrays in Kijimoto et al. (2009; see also Table 1). Genes *phm*, *dib*, *usp*, and *EcR* failed to indicate significant enrichment or depletion in prothoracic horns but nevertheless exist within the *Onthophagus* transcriptome. Lastly, by detecting the expression of active caspase and DNA strand breaks (dashed boxes) the present study implicates additional cardinal components of PCD. (B–D) Example of a failure to remove pupal prothoracic horn in *O. taurus*. (B) Female pupa, (C) corresponding adult, and (D) a female *O. taurus* adult obtained from a laboratory culture (L. W. Simmons, unpublished observation) which failed to fully remove the pupal prothoracic horn primordium and instead molted into a horned female adult.

mechanisms that may coordinate the sex-, species-, and body region-specific resorption of pupal horns. Reanalysis of the microarray data generated in this study allowed us to implicate several putative regulators of PCD-mediated horn resorption in *O. taurus* (Table 1) (reviewed in Yin and Thummel 2005). Among the genes filtered through our analysis, to date only *E93* and *broad* have been shown to be directly involved in PCD in insects (Lee and Baehrecke 2001; Cakouros et al. 2002). It is worth noting that, *broad* possesses many additional functions in development and metamorphosis beyond the regulation of PCD, which may help explain why it is similarly enriched across prothoracic horns, head horns, and legs. The known function of *shade* is to convert ecdysone to its activated state 20-hydroxyecdysone (20E), which is known to play a central role in initiating the molting cycle through the induction of epithelial apolysis (Nijhout 1994). The prothoracic horn-specific enrichment of *shade* may thus result in locally increased 20E in pupal prothoracic epithelial cells causing them to detach from the cuticle. Locally enriched 20E in day1 pupae may also possibly induce the expression of early response genes such as *broad* and *E93*, followed by activation of the caspase pathway to finally execute PCD in day 2 (Fig. 5A). The roles, if any, in the regulation of PCD of the remaining three genes filtered by our analysis remain to be investigated. Experiments are currently under way to test these hypotheses.

PCD and the origin of horns and horn diversity

Recent studies suggest that pupal prothoracic horns function not only as precursors of weapons used by adults but also during the larval-to-pupal molt. Specifically, histological observations suggest that during this transition, prothoracic horns function as molting devices that facilitate the shedding of the heavily sclerotized larval head capsule. This is further supported by experimental manipulations. When the larval

tissue regions that normally would give rise to the pupal prothoracic horn are ablated, animals molt into pupae that lack a prothoracic horn and have failed to shed their larval head capsule (Moczek et al. 2006). Interestingly, all 21 *Onthophagus* species studied in detail so far express pupal prothoracic horns in both males and females, followed by horn resorption in either one or both sexes in each species. Importantly, pupal prothoracic horn resorption and the use of pupal horns as molting devices appear to be restricted to the genus *Onthophagus* and absent in the sister genus *Oniticellus* which lacks any type of horn in both pupal and adult stages. Furthermore, phylogenetic reconstruction of ancestral character states for pupal prothoracic horns across 48 *Onthophagus* species (Emlen et al. 2005) suggest strongly that pupal prothoracic horns arose only once during early *Onthophagus* evolution, possibly to function as molting devices (Moczek et al. 2006). In this context, PCD may have served as an effective developmental mechanism to remove a pupal specific structure in both sexes before the pupal-to-adult molt perhaps in order to recycle resources. If correct, this suggests that *adult* prothoracic horns arose only secondarily, possibly through the sex-specific *inhibition* of PCD in pupal prothoracic horns. Several anecdotal studies suggest that such events do occur in natural populations at least on occasion (Fig 5, B–D; see also Paulian 1935; Ballerio 1999; Ziani 1994).

Possible regulators of PCD during horn resorption

Regardless of the evolutionary history of pupal prothoracic horns, horn resorption is clearly widespread within the genus *Onthophagus*. In most cases, resorption occurs only in females or in both sexes, however, at least one case exists (*O. sagittarius*) in which males, but not females, resorb pupal prothoracic horns, resulting in one of the few, if not the only case of *reversed sexual dimorphism* in the genus (Moczek 2006). This further raises the question as to the nature of the regulatory mechanisms that underlie sex-and species-specific resorption of pupal horns.

Our re-analysis of microarray expression data showed that *broad* and *E93*, two genes known to play important roles in ecdysone-induced PCD in other taxa (reviewed in Yin and Thummel 2005), to be enriched in the prothoracic horns of *O. taurus*, suggesting that an important regulator of PCD in pupal prothoracic horns could be ecdysteroids, similar to the regulation of PCD-mediated pupal wing resorption in tussock moths (e.g., Lobbia et al. 2007). If PCD is executed in the ecdysteroid dosage-dependent manner, genes that affect the local ecdysteroid concentration might be important as well.

Alternatively, *Hox* genes play a critical role in establishing segmental identity, including the segment-specific activation of PCD. For example, the *Hox* gene *deformed* (*dfd*) directly controls the expression of *reaper*, an upstream mediator of PCD, during *Drosophila* mouthpart formation (Lohmann et al. 2002). Similarly, *Ultrabithorax* (*Ubx*) regulates PCD during the formation of the *Drosophila* haltere (Roch and Akam 2000). Interestingly, recent work shows that down regulation of yet another *Hox* gene, *Sex combs reduced* (*Scr*), alters magnitude of sex-specific prothoracic horn resorption in *Onthophagus*, suggesting that PCD genes may be among the targets of *Scr* during prothoracic horn development (Wasik et al. 2010). Lastly, genes normally associated with the patterning of traditional insect appendages (*Distal-less*, *homothorax*) have recently been shown to also regulate horn formation in *Onthophagus* (Moczek and Rose 2009). Another important appendage patterning gene, *decapentaplegic* (*dpp*) regulates PCD during *Drosophila* leg development (Manjon et al. 2007), raising the possibility that appendage-patterning genes could be additional important candidate genes for the regulation of PCD. Lastly, the genes and pathways discussed above may interact to collaboratively regulate and execute PCD in a stage- and location-specific manner.

PCD in nonresorbed horns

Individuals that did not resorb pupal horn primordia and instead retained pupal horns into adulthood, that is the prothoracic horn of male *O. binodis* and the head horns of male *O. taurus*, nevertheless showed signs of at least some PCD occurring during early pupal horn development (Figs. 2 and 4). Hence it is still possible that PCD functions to "sculpt" horns into their final, adult-specific size and shape. For instance, pupal head horns are always slightly larger than the corresponding adult horns they give rise to (Moczek 2007). In addition, subtle shape changes can also be observed during the pupal–adult transition. For instance, the adult horns of males of both species are far more strongly curved and sharply edged than the pupal horns of the same individuals (Fig. 1). In these cases, the main function of PCD may be the fine-tuning of the formation of a future adult weapon, rather than the complete removal of a structure used only during the pupal stage.

CONCLUSIONS

In summary, our results illustrate that PCD plays an important role in the resoprtion of pupal horn primordia. Surprisingly, the degree of PCD mediated horn resorption depended strongly on species, sex, and body region, suggesting the existence of regulatory mechanisms that can diversify quickly over short phylogenetic distances. More generally, our results illustrate that secondary, differential loss of structures during development can be a powerful mechanism for generating diversity within and between species.

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