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Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae)

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Abstract

Male dung beetles (*Onthophagus taurus*) facultatively produce a pair of horns that extend from the base of the head: males growing larger than a threshold body size develop long horns, whereas males that do not achieve this size grow only rudimentary horns or no horns at all. Here we characterize the postembryonic development of these beetles, and begin to explore the hormonal regulation of horn growth. Using radioimmune assays to compare the ecdysteroid titers of horned males, hornless males, and females, we identify a small pulse of ecdysteroid which is present in both hornless males and females, but not in horned males. In addition, we identify a brief period near the end of the final (third) larval instar when topical applications of the juvenile hormone analog methoprene can switch the morphology of developing males. Small, normally hornless, males receiving methoprene during this sensitive period were induced to produce horns in 80% of the cases. We summarize this information in two models for the hormonal control of male dimorphism in horn length.

Keywords: Horned beetle; Polyphenism; Male dimorphism; Threshold; Juvenile hormone

1. Introduction

Males in many species of Scarabaeid beetles have horns. Beetle horns appear to have arisen through intense sexual selection, and function as weapons during intermale combat over reproductive access to females (Beebe, 1944; Palmer, 1978; Eberhard, 1979, 1987; Siva-Jothy, 1987; Goldsmith, 1987; Conner, 1988; Emlen, 1994, 1997a). In some horned beetle species not all males produce horns, and the hornless males frequently employ non-aggressive alternative behavioral tactics to encounter and mate with females (Eberhard, 1982; Goldsmith, 1987; Cook, 1990; Rasmussen, 1994; Emlen, 1994, 1997a; Moczek, 1996).

Males of the dung beetle *Onthophagus taurus* Schreber (Coleoptera: Scarabaeidae) wield a pair of curved horns that extend from the base of the head (Fabre, 1899). The lengths of these horns scales positively with male body size, so that large males have disproportionately longer horns than small males. Natural populations of *O. taurus* are characterized by a broken, or 'triphasic' scaling relationship between horn length and body size (Fig. 1): males growing larger than a critical, or threshold body size (in this case, 4.9 mm prothorax width) produce fully developed horns; males not attaining this body size produce only rudimentary horns, or no horns at all. Females in this species never produce horns.

Horn development is facultative, and depends on the the attainment of a certain body size, which, in turn, depends on the nutrient conditions encountered by males during development (Emlen, 1994, 1997b; Moczek, 1996; Hunt and Simmons, 1997; Moczek and Emlen, in press). Experimental manipulation of food quantity significantly influences the horn morphology of males: male larvae provided with a larger amount of food develop into adults with long horns, whereas males reared on reduced food amounts do not develop horns, irrespective of the morphology of their fathers (Emlen, 1994; Hunt and Simmons, 1997). The facultative production of horns suggests that male dimorphism in this species may be regulated hormonally, in a fashion simi-

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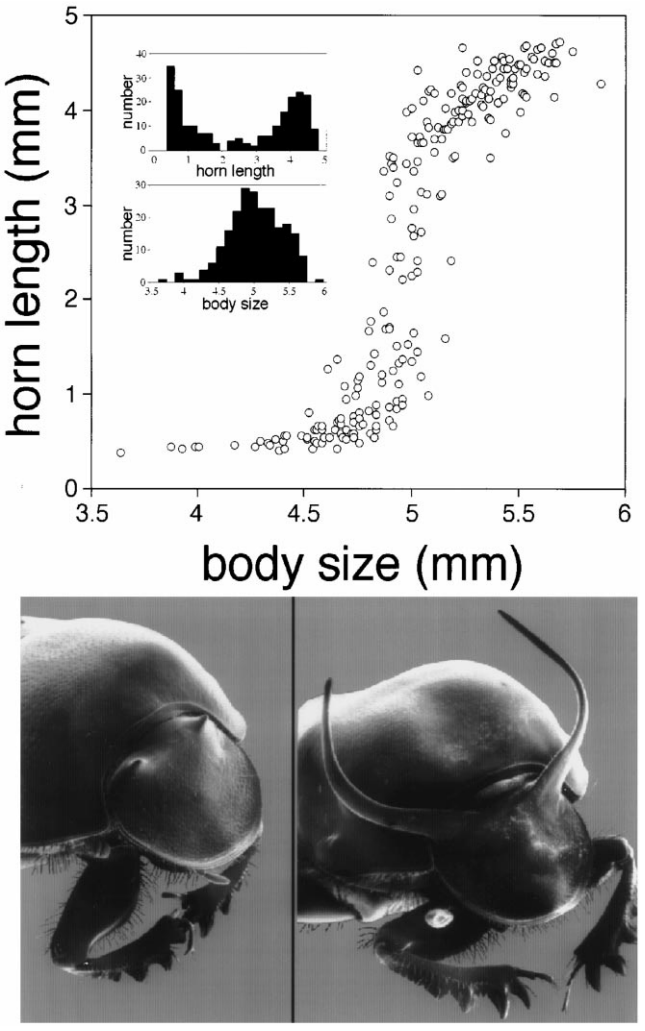

Fig. 1. Scaling relationship between horn length and body size (prothorax width) for 300 male *Onthophagus taurus* (Coleoptera: Scarabaeidae) sampled from pastures in Durham County, North Carolina. The sigmoidal relationship is associated with a bimodal frequency distribution of horn lengths in natural populations (top insert), and separates males into hornless and horned adult shapes.

lar to the regulation of winged or wingless adult forms in aphids (Lees, 1977; Hardie, 1980, 1987), and crickets (Zera and Tiebel, 1989; Zera and Tobe, 1990; Tanaka, 1994), social castes in Hymenoptera (Wirtz, 1973; Wheeler and Nijhout, 1981, 1983; Rachinsky and Hartfelder, 1990; Wheeler, 1991), and Isoptera (Lenz and Westcott, 1985) and seasonal wing pattern polyphenisms in Lepidoptera (Endo and Funatsu, 1985; Endo and Kamata, 1985; Koch and Bückman, 1987; Rountree and Nijhout, 1995).

Here we characterize the post-embryonic development of *O. taurus*. First we develop a method for staging developing larvae based on several characteristic morphological and behavioral transitions. Using this information, we measure ecdysteroid titer profiles for horned males, hornless males and females at each of the identifiable developmental stages, and quantify the effects on metamorphosis of topical applications of the juvenile hormone (JH) analog methoprene at each of these same stages. Finally, we identify a sensitive period near the end of larval development during which topical application of methoprene induces production of horns. These findings are summarized in two simple models for the timing and endocrine regulation of horn growth in this species.

2. Methods

2.1. Rearing beetles

Beetles were collected from horse pastures in Durham County, North Carolina. Pairs of adults were placed in cylindrical buckets (10 cm diameter × 30 cm) three-quarters filled with a moist sand/soil mixture and provided with unlimited horse manure (methods described more fully in Moczek, 1996, Moczek and Emlen, in press). Beetles excavated tunnels into the soil, and pulled pieces of dung below ground to fashion dense, cylindrical brood masses, each containing a single egg. Every four days the soil in each of the buckets was sifted, and all brood masses removed and placed in separate soil-filled cups and stored at 28°C and 80% humidity for the duration of larval development.

When larvae molted into the third (final) instar (mean ± SD = 7.8 ± 1.3 days after oviposition) they were transferred to containers that permitted direct observation and removal for weighing. Observation containers were made from plaster of paris blocks with a 1 cm diameter and 2 cm deep hole drilled into the center. Holes were partially filled with horse manure, and covered with microscope coverslips using stopcock grease. Cubes were moistened daily with a spray-mister and kept in sealed, aluminum foil-covered plastic boxes lined with fungicide-treated paper (1% sorbic acid solution).

Three hundred *O. taurus* larvae were observed and weighed daily to characterize the development and growth trajectories of horned males, hornless males and females. Five morphologically and behaviorally distinguishable stages were identified. Stages I through III comprise the feeding period of the third (final) instar, and are recognized by transitions of the integument from clear to opaque, of the dorsal vessel from very thin to large and dark, and by an increasing deposition of fat body so that the larva becomes first mottled, and then an opaque white/yellow. During all three of the feeding stages, larval exudate is extensive and black. Stage IV animals purge their gut. In purging animals the dorsal vessel is conspicuously absent (a clear channel along the otherwise opaque white/yellow dorsum), the anal exudate is a light brown early in stage IV and clear by the end of stage IV, and the abdomen becomes increasingly smaller and more compact. Stage V animals have com-

pleted their gut purge (prepupa stage), are completely opaque, very compact, and have no exudate. Early stage V animals contract their abdomen frequently, whereas later stage V animals are almost completely motionless. The average ages of animals at each of these stages are presented in Table 1. Physiological age based on these five stages, rather than chronological age, was used for all subsequent experiments.

2.2. Ecdysteroid radioimmune assay

To characterize the ecdysone titer profile of horned males, hornless males and females of this species, we performed a radioimmune assay for ecdysone using the methods of Borst and O'Connor (1972) and Warren et al. (1984). Up to 10 μl haemolymph was extracted from each larva by puncturing the abdominal cuticle with a glass needle and collecting the droplet in a capillary tube. Haemolymph samples were immediately blown into 100 μl chilled 100% methanol and vortexed. Samples were centrifuged for 3 min at 13 000 RPM, the supernatant transferred to labelled Eppendorf tubes, dried in a vacuum centrifuge, and stored until needed at $-$ 20°C. When it was not possible to obtain 10 μl from an animal, smaller amounts were used, and final ecdysone counts converted to amount per 10 μl.

2.3. Methoprene application

To identify the effects of augmented levels of JH on the timing of metamorphosis, as well as its possible effects on male horn determination, we topically applied the JH analog methoprene to developing larvae. Methoprene was dissolved in acetone to a concentration of 10 μg/ml. Five microliters of this solution were applied to each larva, achieving an approximate dose of 400 μg/g larval weight. Two hundred and seventy one larvae were reared as described above. Larvae of each age were divided randomly among either a methoprene treatment or an acetone-treated control. Methoprene or acetone was administered topically to the dorsum of each larva, immediately behind the head capsule. Doses were applied slowly to prevent loss from run-off or damage to larvae from solutions entering spiracles. Larvae were observed and weighed daily for the remainder of their development, and the number of days to pupation recorded, as well as the weight, sex and horn morphology of pupae.

3. Results

3.1. Development of Onthophagus taurus

Onthophagine beetles pass through three larval instars and pupate within the confines of a brood mass—a finite, oval mass of dung buried by the female parent. When larvae are transferred from natural brood masses to artificial ones made from plaster, their behavior as well as morphology can be observed. Third (final) instar larvae exhibited five distinct morphologically and behaviorally recognizable stages (see above, and Table 1). The first three of these stages comprise a feeding period, followed by a two day gut-purge. As the animals purged their guts they fashioned a shell made from dung and anal exudate around themselves in the center of the brood mass (these pupal cells are described by Main, 1922, and Halffter and Edmonds, 1982). After completion of the pupal cell and the gut purge, animals are morphologically much more compact, and become increasingly immobile (prepupa stage), before molting into pupae. The pupal stage lasts 8.65 $\pm$ 0.95 days at 28°C, and adults dig to the soil surface after eclosion.

We found no evidence to indicate that these beetles employ a critical body size as a proximate cue for initiating metamorphosis (as in Wigglesworth, 1970; Nijhout and Williams, 1974a; Nijhout, 1975, 1981). Instead, metamorphosis begins soon after the larva runs out of food. For example, transferring larvae to artificial brood balls lacking dung resulted in larvae initiating their gut purge within approximately two days, regardless of the size or weight of the larva at the time of transfer (provided they were all in their final instar).

Male horns first appear at the end of the final larval instar as a pair of invaginations of the larval epidermis in the occipital region of the head—inside the larval head capsule. In males destined to produce long horns, these 'pockets' of epidermis grew rapidly over a two day per-

Table 1
Physiological stages of *Onthophagus taurus*

Stage	Behavior	Duration (days $\pm$ SD)	N
egg, first and second instars		7.8 $\pm$ 1.3	66
third instar: I	feeding	7.0 $\pm$ 2.5	82
II	feeding	1.8 $\pm$ 1.3	62
III	feeding	1.0 $\pm$ 0.6	65
IV	gut purge	1.6 $\pm$ 0.6	71
V	prepupa	1.6 $\pm$ 0.5	78
pupa		8.7 $\pm$ 1.0	66

iod coinciding with the latter half of the gut purge (stage IV), to produce bulging masses of highly folded epidermis (Fig. 2). These telescoped horn precursors remained trapped inside the larval head capsule during the prepupal period (stage V), and expanded to form the fully extended horns at pupation.

3.2. Ecdysteroid titers

Hormone titer profiles for ecdysone in horned males, hornless males and females are presented in Fig. 3a. Beetles exhibited a sharp rise in haemolymph levels of ecdysteroids late in the third instar, immediately preceding the gut purge (stage IV), with levels continuing to rise throughout the gut purge and prepupal periods (stage V). Females also exhibited a small, distinct pulse in haemolymph levels of ecdysteroid during the feeding period, prior to the main peak in hormone level (Fig. 3a). Interestingly, hornless (but not horned) males also exhibited this earlier peak in haemolymph ecdysteroid level (Fig. 3a), revealing a sex-and morph-specific difference in ecdysteroid titer.

3.3. Methoprene applications

3.3.1. Effect on metamorphosis

Topical application of the JH analog methoprene to feeding larvae (stages I–III) significantly delayed the onset of pupation, as compared with acetone-treated control animals (Fig. 3b). After the initiation of the gut purge (stage IV), however, metamorphosis appeared irreversible, as topical applications of methoprene no longer delayed the onset of pupation (Fig. 3b). These results are consistent with endocrine models for control of metamorphosis in other holometabolous insects (Truman et al., 1974; Nijhout and Williams, 1974b; Bollenbacher et al., 1981; Nijhout, 1981, 1994; Gilbert et al., 1996), including Coleoptera (Delbecque et al., 1978; Besson-Lavoignet and Delachambre, 1981; Connat et al., 1984, 1991; Quennedey et al., 1995): the presence of JH in the haemolymph during the feeding period of the last larval instar inhibits both PTTH and ecdysteroid secretion (Nijhout, 1975, 1994; Rountree and Bollenbacher, 1986; Bollenbacher, 1988). Once PTTH and ecdysteroid secretion has been initiated, the process of metamorphosis becomes irreversible, and subsequent augmentation of haemolymph levels of JH no longer affect the timing of the metamorphic molt. This is consistent with the finding that the physiological stage at which metamorphosis becomes irreversible in *Onthophagus* coincides with the sharp rise in haemolymph titers of ecdysone (Fig. 3).

3.3.2. Determination of male horn morphology

Topical applications of methoprene during the first half of the gut purge (stage IV) significantly affected the horn morphology of developing males. Application of methoprene to small males (who would typically develop without horns) induced these males to switch from hornless to horned development in 80% of the cases (Fig. 4). Although application of methoprene to late 3rd instar larvae (stages IV and V) invariably caused these animals to die at pupation (they appeared unable to shed the old larval cuticle), telescoped horns were clearly recognizable as folded structures within the larval head capsules (as in Fig. 2). Similar application of methoprene to large males (who typically develop with horns) did not switch these males to hornless development (Fig. 4); nor did application of methoprene to females induce these animals to produce horns.

These results suggest the existence of a critical period of JH sensitivity for horn induction late in the third larval instar, after metamorphosis has become irreversible, during the first half of the gut purge stage. Application of JH during this window affects the horn morphology of developing males. The timing of this critical period for

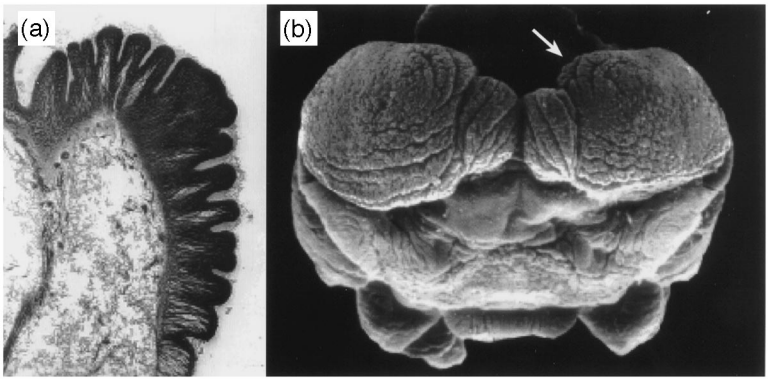

Fig. 2. Developing horns in male *Onthophagus taurus*. Horns start as a regional detachment of the epidermis from the larval cuticle. This epidermis grows rapidly over a 24–48 hour period, but is trapped beneath the head capsule as it grows, and subsequently forms a dense series of telescoped, concentric folds. (a) Cross-section of a developing horn, with larval head capsule removed (late stage IV; animal is facing to the right). By the end of the larval period (stage V), the two horns are visible beneath the head capsule as bulges of folded epidermis (b); front view, shown with head capsule removed.

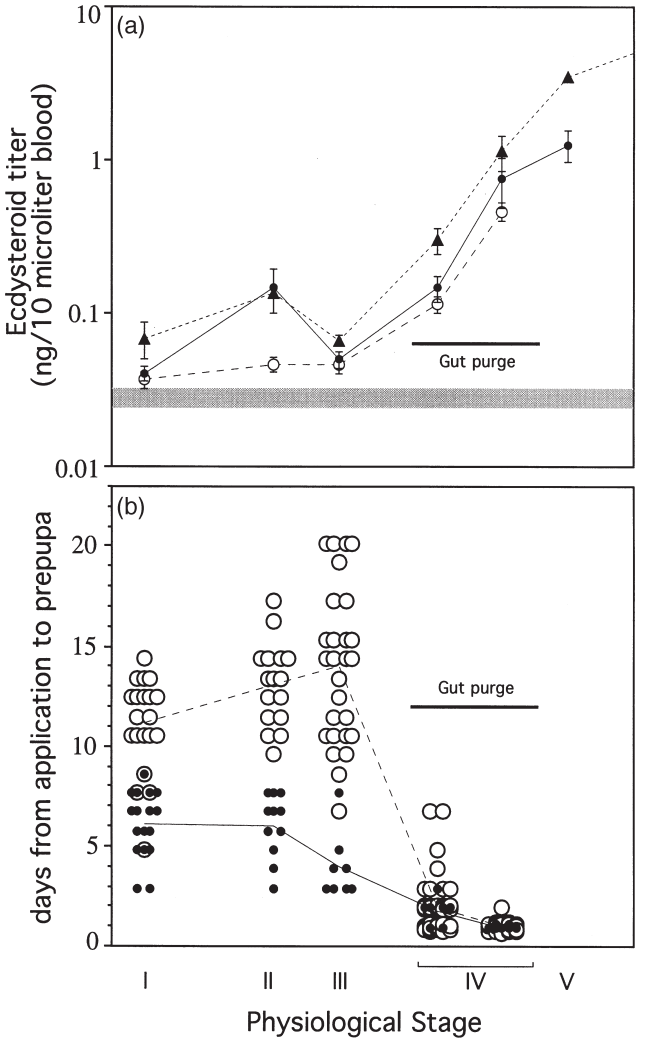

Fig. 3. Hormonal changes in the haemolymph of developing *Onthophagus taurus*, as inferred from ecdysteroid radioimmune assays and topical applications of methoprene. (a) All animals ($n = 220$) exhibited an increase in ecdysteroid titers immediately preceding the onset of the gut purge, and continuing through pupation (note log scale). Females (▲) and hornless males (●) also showed an earlier, smaller pulse in ecysteroid titers during the feeding stage (stage II), which was not present in horned males (○). Gray bar indicates minimum detectable levels (non-specific binding in tubes with no antibody added). (b) Animals receiving topical applications of the juvenile hormone analog methoprene (○) delayed pupation, as compared with acetone-treated control animals (●), when methoprene was applied during the feeding period (stages I–III). However, the timing of pupation was not affected by methoprene once animals had initiated their gut purge (stage IV).

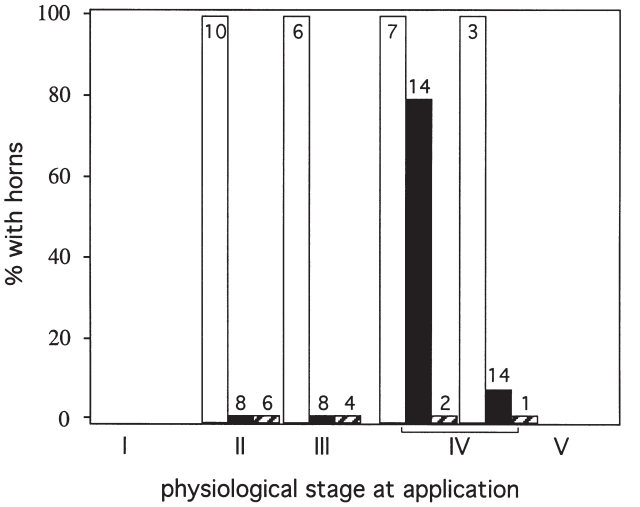

Fig. 4. Effect of topical applications of methoprene on the horn morphology of developing male *Onthophagus taurus*. Large males (open bars) produced horns irrespective of hormone treatment (methoprene and acetone control animals combined). Small, normally hornless, males (closed bars) could be induced to produce horns when methoprene was added during the first half of the gut purge (stage IV), but not at other times. Acetone applications to small males (dashed bars) never induced horn growth. Numbers of individuals are indicated above bars.

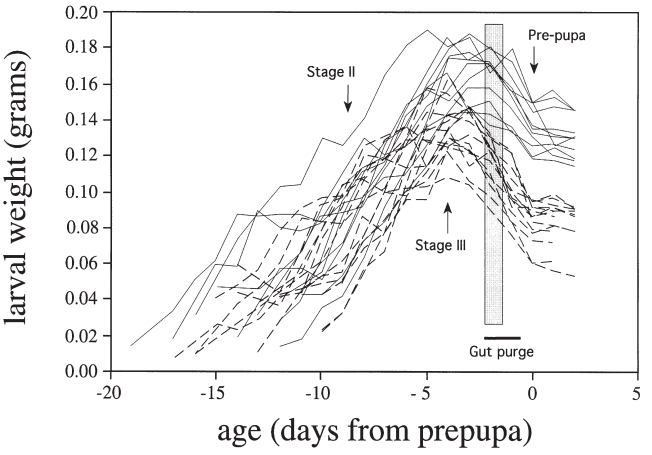

Fig. 5. Final instar growth curves for horned (solid lines) and hornless (broken lines) male *Onthophagus taurus*, showing the critical period for horn determination (gray bar). During this critical period, small (normally hornless males) can be induced to produce horns by the topical application of methoprene (see Fig. 4).

horn determination is also implied by the growth curves of individual males (Fig. 5). Growth trajectories of presumptive horned and hornless males only became distinguishable very late in larval life—after the onset of the gut purge, and coincident with the critical period of Fig. 4.

4. Discussion

The experiments described above suggest that the endocrine regulation of metamorphosis in *O. taurus* is similar to general models described for other holometabolous insects. The presence of JH during the final larval instar inhibits the initiation of metamorphosis, and applications of JH significantly delay the onset of pupation (Fig. 3b). In other insects, a drop in JH titer disinhibits the secretion of PTTH and ecdysteroids.

In most insects studied, attainment by a larva of a critical size or weight is responsible for preventing subsequent JH release. These so-called "critical sizes for metamorphosis" have been characterized in a diverse array of taxa, from Lepidoptera to social Hymenoptera. In contrast, *O. taurus* larvae appear to employ a different stimulus—in this case, metamorphosis is triggered when the larva runs out of food. Larvae reared on reduced food amounts pupate sooner, and at a smaller body size, than larvae with larger food amounts (Emlen, 1994; Moczek, 1996; Hunt and Simmons, 1997). Similarly, transfer of final instar larvae to artificial brood chambers lacking food leads to pupation approximately four days later, irrespective of their age or body size at the time of transfer.

On reflection, this departure from the norm seems intuitive. Insect larvae who are either supplied continuously with food (e.g. social insect larvae), or who are mobile, so that they can seek forage as needed (e.g. tobacco hornworm caterpillars in the field), may benefit by incorporating a critical body size or weight for metamorphosis. Given the clear reproductive advantages frequently associated with large body sizes (e.g. Thornhill and Alcock, 1983), such a mechanism would effectively prevent premature attainment of sexual maturity, and ensure that all individuals reach a competitive body size. In this situation, larvae feed for as long as is needed to attain the necessary body size, sometimes even undergoing additional larval molts if the rate of growth is inadequate (Nijhout, 1994).

In *O. taurus* and many other Scarabaeids, by contrast, larvae develop within isolated brood masses—finite aliquots of diet that are buried by the parent females. Larvae cannot seek additional resources, and must develop completely using only the amount of food available. Species incapable of attaining adult (reproductive) status without reaching a critical body size would suffer a tremendous cost whenever the food available was insufficient to permit their larvae to reach the critical size. Therefore, evolution of an alternative cue for the initiation of metamorphosis seems reasonable. In this case, larvae that can complete development at a variety of body sizes probably reproduce under a broader range of circumstances than larvae needing to first attain a critical body size.

Yet having a large body size is no less vital to reproductive success in these beetles than it is anywhere else (see e.g., Cook, 1990; Rasmussen, 1994; Emlen, 1994, 1997a, b; Moczek, 1996). Again, using the depletion of food as a cue makes biological sense. Larvae that wait to initiate metamorphosis until all available food has been consumed will attain as large a body size as possible, yet still be capable of completing development under a broad range of nutritional circumstances.

It will be interesting to see whether similar cues initiate metamorphosis in other dung beetle species. Many dung beetles (e.g. Aphodius) complete their life cycle inside dung pats. In these species, because larvae are free to move around inside the dung, they may be able to continue to feed until they attain a critical body size for metamorphosis (although competition for dung resources is often intense, and larval growth may still be unpredictable; Cambefort and Hanski, 1991). However, dung beetles that remove pieces of dung from the source pat, either by pulling it into tunnels (e.g. Scarabaeinae) or by rolling it away and burying it later (e.g. Coprinae), should all face the same situation as *O. taurus*: larvae must complete their development using only the finite amount of dung provided by the parent beetles. It is in these taxa that we might expect to find food-depletion cues involved with the completion of development.

Comparison of ecdysteroid profiles revealed one interesting difference between horned and hornless morphs. Although overall the profiles were very similar, with a sharp rise in ecdysteroid late in the third instar, there is a morph-specific difference with a peak in ecdysteroid levels in females and hornless males during physiological stage II (Fig. 3a), prior to the gut purge, and long before the JH-critical period for horn determination. This peak in ecdysteroid level occurs at a time when the growth curves of the sexes and male morphs are indistinguishable (Fig. 5), raising the question of how early in development animals "decide" whether they will be horned or hornless. All evidence points to this "decision" occurring later in the larval period, when differences in body size serve as an appropriate cue for the switch (Fig. 5), and JH clearly affects the regulation of horn growth (Fig. 4).

It is worth noting that hornless males and females are very similar morphologically. Females never produce horns, and in many respects, hornless males have converged on a 'female-like' shape. Our results raise the possibility that the earlier peak in ecdysteroids might be involved somehow in the production of a female-like morphology, thus explaining its presence in females and hornless males, but not in horned males. Clearly, the biological significance of this early peak in ecdysone needs to be explored further.

In this paper we identify a critical period for horn determination. During this period, topical applications of JH can cause presumptive hornless males to switch to horn production, a developmental pathway inappropriate for their body size (Fig. 4). Two mechanisms might account for this effect of JH. (1) A morph-specific difference in JH levels could drive the dimorphism, either through differences in JH production or JH breakdown (Zera and Tiebel, 1989; Zera and Tobe, 1990), so that large males have higher levels of JH during the critical period than smaller males, or (2), JH levels may be similar for large and small males, but the morphs instead differ in the sensitivity of their tissues to this level of JH. Consequently, we summarize our results in not one,

but two possible endocrine models for the control of horn dimorphism in beetles (Fig. 6), which differ in the mechanism by which JH levels are translated into commitment by a male to either a horned or a hornless morphology.

Although there are many similarities between horn length diphenism in beetles, and worker-soldier diphenism in ants (e.g. Wheeler and Nijhout, 1983; Wheeler, 1986, 1991), the critical period for morph determination in beetles occurs much later in development than that found in the ant, *Pheidole bicarinata* (Wheeler and Nijhout, 1983; Wheeler, 1991). The relatively late determination of male morphology in *O. taurus* seems reasonable, given the natural context of this dimorphism. Beetle horns are used as weapons in inter-male combat over reproductive access to females (Beebe, 1944; Palmer, 1978; Eberhard, 1979, 1987; Siva-Jothy, 1987; Goldsmith, 1987; Conner, 1988; Emlen, 1994; Moczek and Emlen, in press). As with most other secondary sexual traits, the functional significance of beetle horns is very closely tied to body size: males benefit by producing the largest horns that they can effectively wield, but their effectiveness at using horns depends critically on how large they themselves grow to be (Nur and Hasson, 1984; Kodric-Brown and Brown, 1984; Zeh and Zeh, 1988). In this situation, where the final body size of a growing male is unpredictable (as compared with an insect that uses a critical body size for initiating metamorphosis; see above), a very late period of horn length determination may provide the most effective match between male horn morphology and male body size, because the "decision" of whether or not to produce horns would occur after all growth in body size has ceased.

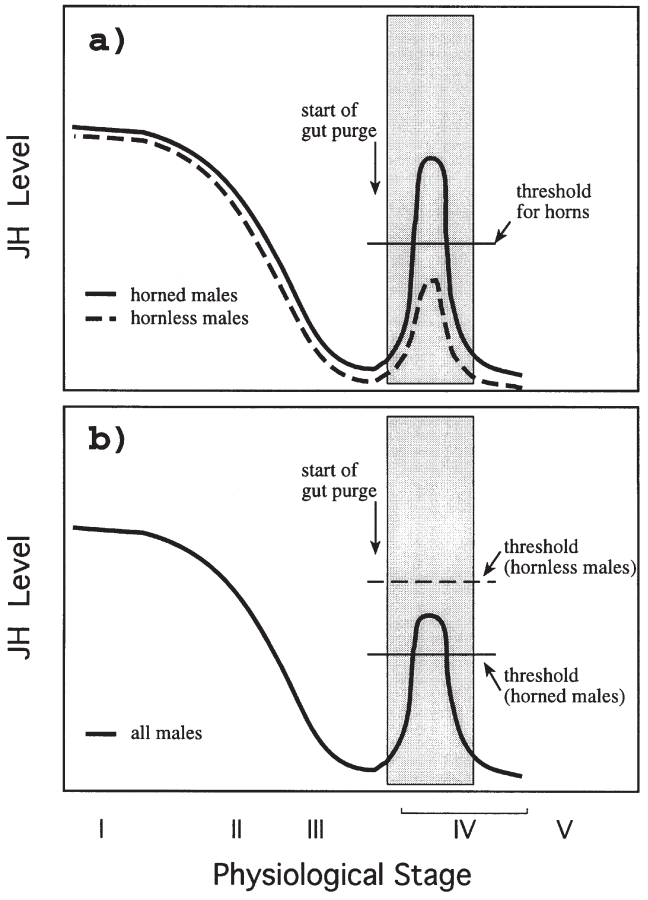

Fig. 6. Alternative hypotheses for the hormonal control of male horn dimorphism in the beetle *Onthophagus taurus*. After levels of JH have declined sufficiently to initiate the gut purge and metamorphosis, a subsequent pulse of JH determines the horn morphology of males. (a) During the first half of the gut purge (stage IV), large and small males differ in their concentration of JH, such that large males have higher concentrations than smaller males. Concentrations of JH higher than a threshold level induce males to produce horns. (b) Same as in (a), except that all males have similar levels of JH during the horn-determining sensitive period, and large and small males instead differ in their sensitivity to JH, so that males larger than a threshold size respond to the JH, while smaller males do not. Each of these models could explain the observation that augmented levels of JH during this sensitive period cause small, normally hornless, males to switch to horn production.

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