Juvenile Hormone Mediates Sexual Dimorphism in Horned Beetles

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ABSTRACT The causes and consequences of sexual dimorphism are major themes in biology. Here we explore the endocrine regulation of sexual dimorphism in horned beetles. Specifically, we explore the role of juvenile hormone (JH) in regulating horn expression in females of two species with regular sexual dimorphism for pronotal horns (females have much shorter horns than males) and a third species with a rare reversed sexual dimorphism for both pronotal and head horns (females have much larger horns in both body regions compared with males). Applications of the JH analog methoprene caused females of the two more typical species to grow significantly shorter pronotal horns than control females, whereas no consistent effect on pronotal horn development was detected in the third, sex-reversed species. Instead, females in this species showed an unexpected and significant increase in head horn expression in response to methoprene treatment. Lastly, horn shape was also affected in females of one of the regularly sexually dimorphic species, but in the opposite direction than horn lenght. Although methoprene exerted a feminizing effect on female horn length in this species, it significantly masculinized horn shape by inducing a peculiar shape change observed naturally only in males. Our results suggest that JH influences both overall size and shape of female horns, but does so flexibly and as a function of species, sex and horn location. We use our results to review current models on the role of endocrine mechanisms in development and evolution of horned beetle diversity. *J. Exp. Zool. (Mol. Dev. Evol.) 308B, 2007.*

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The origin and maintenance of sexual dimorphism represent major themes in evolutionary biology (Andersson, '94; Shuster and Wade, 2003), and the evolutionary forces that shape sexual dimorphisms have been studied extensively in a wide range of organisms and on a variety of levels of biological organization (e.g., Delph, 2005; Fairbairn, 2005; Ketterson et al., 2005). More recently, research has ultilized a developmental perspective with the goal to better understand how ontogenetic processes facilitate or limit the expression of sexual dimorphism and mediate evolutionary divergence in degree and kind of sexual differences. Developmental studies have firmly established hormonal control mechanisms as powerful proximate regulators in the development of sex differences. More recently, hormones and their associated pathways have also emerged as important mediators of evolutionary changes in the degree and nature of sexual dimorphism, able to facilitate, constrain, or bias evolutionary trajectories depending on the exact nature of the underlying endocrine control mechanisms (Ketterson et al., 2005; Emlen et al., 2005a; Navara et al., 2006). Here we explore the role of an important insect hormone, juvenile hormone, in the development and evolution of sexual dimorphisms in size and shape of secondary sexual traits in horned beetles.

Horned beetles, and in particular the genus *Onthophagus*, have become an attractive study system to explore how development and evolution interact in the origin of phenotypic diversity (reviewed in Emlen, 2000; Moczek, 2005, 2006a,b). Dramatic diversity in horn expression exists both

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above and below the species level, and environmental and genetic contributions to phenotypic variation differ across these levels. Genetic differences account for often dramatic differences in horn shape, number of horns grown, and exact location of horn expression observed between species (Arrow, '51; Balthasar, '64; Matthews, '72). Genetic differences also account for widespread sexual dimorphism within the majority of species (Emlen et al., 2005a,b). Sex in almost all families of beetles, including the Scarabaeidae, is chromosomally determined (XY vs. XX) with males representing the heterogametic sex (Smith and Virkki, '78; Galian et al., 2002; Bione et al., 2005). In almost all species only males express fully developed horns while females express no or greatly reduced horns (Balthasar, '64). Intraspecific variation is similarly extreme within the male sex, though here environmental factors are responsible for most of the observed phenotypic diversity. Only male larvae that have access to optimal feeding conditions eclose above a critical adult size threshold and develop a full set of horns as adults, whereas male larvae with access to sub-optimal feeding conditions eclose to a smaller adult size and remain more female-like and largely hornless (Emlen, '94; Hunt and Simmons, '97; Moczek, '98; Moczek and Emlen, '99). Lastly, the exact scaling relationship, or allometry, between male horn length and body size is affected subtly by environmental conditions (Emlen, '97; Moczek, 2002), but can also diverge genetically between conspecific populations, sometimes generating heritable allometric divergences between conspecific populations that are similar in kind and magnitude to those observed between species (Moczek and Nijhout, 2002, 2003; Moczek, 2003). This study focuses on the developmental regulation of (i) sexual dimorphisms in thoracic horn expression and (ii) interspecific differences in degree and kind of sexual dimorphism.

Growth and development of beetle horns

Growth and development of beetle horns have so far only been studied in detail in the dung beetle genus *Onthophagus*. The actual growth of horns is an extremely dynamic process confined to an approximately 48-hr window of time immediately before the transition from larva to pupa (Emlen and Nijhout, '99; Moczek and Nagy, 2005). During this stage, also referred to as the *prepupa*, selected epidermal regions detach (apolyse) from the cuticle and undergo rapid cell proliferation. The resulting tissue then becomes compacted and folded underneath the larval cuticle. During the second half of the prepupal stage the epidermis secretes the future pupal cuticle. Upon pupal ecdysis the folded horn tissue is permitted to telescope out and form the future pupal horn, and the pupal cuticle hardens within hours of eclosion. All species investigated thus far share this period of prepupal horn growth. It is also at this prepupal stage that *differences* in prepupal horn growth generate many of the differences in horn expression we see in adults. For example, in *O. sagittarius* (Fig. 1C) only large adult females express a single, large medial head horn whereas adult males express a pair of small head horns near the front of the head. These differences in adult horn expression are already clearly established in pupae and thus are due to differences in initial horn growth during the prepupal stage. However, this is not true for all species, nor is it true for all horn types (Moczek and Nagy, 2005).

A second developmental period critical to defining the final size and shape of adult horns occurs during the pupal stage. In *Onthophagus* this stage lasts from one to several weeks depending on species. During this stage the animal undergoes principally the same basic developmental steps as during the previous larval stage, including

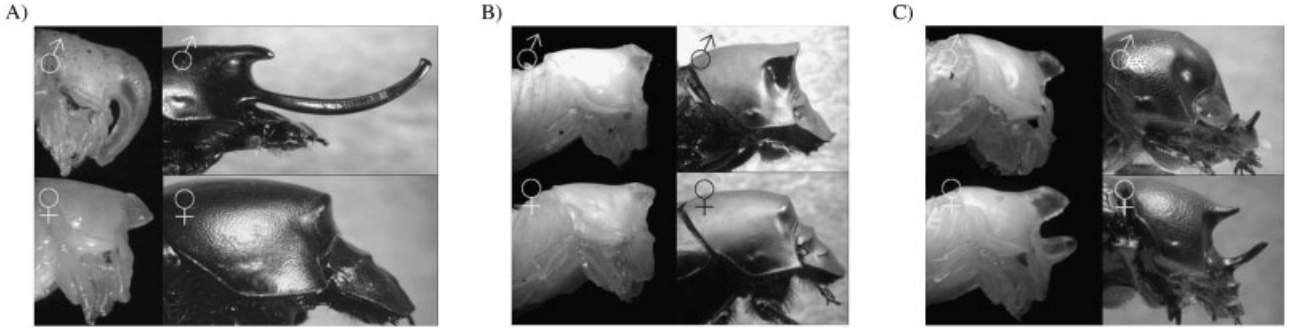

Fig. 1. Species used in the present study. (**A**) *Onthophagus nigriventris*, (**B**) *O. binodis*, and (**C**) *O. sagittarius*. Shown are males (top row) and females (bottom row) as pupae (left) and corresponding adults (right).

apolysis of the epidermis, secretion of a new cuticle, and eclosion to the next, now adult, stage. The main differences with respect to horn development are twofold: First, there is no major epidermal proliferation stage. Horns, just like other body parts, do not grow significantly during the pupal stage. Secondly, in at least one horn type, those extending from the thorax, there is frequent differential loss of presumptive horn tissue, most likely through programmed cell death. This process is highly developmentally flexible and evolutionarily labile and can magnify, reverse, and erase sexual dimorphism generated through differential prepupal growth (Moczek, 2006b). In species in which prepupal growth is sexually monomorphic, this process alone is sufficient to create sexual dimorphism, allowing sexually monomorphic pupae to eclose into sexually dimorphic adults. Here we explore the endocrine regulation of horn growth in females of three *Onthophagus* species that differ markedly in the relative contributions of prepupal growth and pupal resorption to sexual dimorphism.

Endocrine regulation of horns in male beetles

Earlier studies have implicated juvenile hormone (JH) as an important regulator of horn expression in male *Onthophagus* beetles. JH, is a sesquiterpenoid derived from farnesenic acid and is involved in one way or another in almost every aspect of insect development and reproduction, including molting and metamorphosis, ovarian development and vitellogenin synthesis, caste determination in social insects, and polyphenic development in locusts, aphids, and honey bees (Nijhout, '94). In *O. taurus,* ectopic applications of the synthetic JH analog methoprene induced the expression of horns in males that normally would have developed into small hornless males, provided exposure to methoprene took place during a particular sensitive period late in larval development, approximately 24–48 hr before the gut purge (Emlen and Nijhout, '99). On the basis of this observation Emlen and Nijhout ('99) postulated that male larvae may differ in their JH titers depending on their body weight, and that there is a brief sensitive period during late larval development during which JH titers serve as a proxy for future adult body size. Under this model only male larvae heavy enough to express JH titers that exceed a certain titer threshold during this sensitive period will develop into horned males, whereas those exhibiting small body mass, and consequently, titers below the threshold, will become hornless. A subsequent study confirmed the existence of this late JH sensitive period, and, more importantly, showed that evolutionary changes in degree and timing of sensitivity to JH have provided important avenues for evolutionary changes in patterns of horn expression in different geographically isolated populations exposed to divergent ecological conditions (Moczek, 2003; Moczek and Nijhout, 2003). Combined, these observations raised the possibility that JH may also be involved in regulating the absence of horns in female *Onthophagus*, and that evolutionary changes in this regulation may have given rise to the varying degrees of sexual dimorphism observed between species. Here we explore the role of JH in the regulation of female horn length and horn shape in three closely related *Onthophagus* species.

MATERIAL AND METHODS

Species choice

We studied the role of JH in development of horns in females in three *Onthophagus* species that rely at least in part on different developmental mechanisms in generating sexual dimorphism (Moczek, 2006a,b, 2007). In *O. nigriventris* (Fig. 1A) differential growth of thoracic (pronotal) horn primordia during the prepupal stage generates sexually dimorphic pupae, expressing a large down-curved horn in male and only a moderate horn in female pupae. Sexually dimorphic prepupal growth is then followed by sexually dimorphic resorption of pupal horn tissue in female but not male *O. nigriventris*, resulting in a dramatic sexual dimorphism among adult *O. nigriventris*. In this species adult sexual dimorphism is therefore the sum of two discrete developmental processes; differential prepupal growth generates sexually dimorphic pupae, and subsequent greater resorption of pupal horns in females compared with males further magnifies this dimorphism into the adult stage (Moczek, 2006b). In contrast, in *O. binodis* initial prepupal growth of pronotal horns is identical in both sexes and generates sexually monomorphic pupae with respect to horn length (Fig. 1B; Moczek, 2006b). Uniform prepupal growth is then followed by massive resorption of pupal horns in females but retention of horns in all males, giving rise to a relatively moderate sexual dimorphism among adult *O. binodis*. In this species, differential resorption of horn tissue in females but not males

is therefore the sole mechanism responsible for generating horn length dimorphism among the sexes. The same pattern can be observed in the third species, *O. sagittarius*, except in a sex-reversed manner (Fig. 1C). Although initial prepupal growth of pronotal horns is nearly identical in both sexes, in this species only female pupae retain their pronotal horn into adulthood whereas male pupae resorb their pronotal horn before the final molt, creating one of the rare reversed sexual dimorphisms in the genus *Onthophagus*. In addition, and unlike the previous two species, *O. sagittarius* also expresses a second horn type: head horns (Fig. 1C). Females express a single, large, medial horn protruding from the center of the head, whereas males express a pair of small horns near the periphery of the head. Unlike in thoracic horns, little pupal resorption occurs in head horns and sex-specific differences in adult horn expression are primarily, if not exclusively, the product of differential prepupal growth.

Beetle husbandry and hormone treatments

O. nigriventris and *O. binodis* were collected from pastures near Waimea, Hawaii, and *O. sagittarius* was collected from pastures near Sunset, Oahu. All three species were established in the laboratory and maintained and reared as described in Moczek and Nijhout (2003). Early third instar larvae of both species were transferred from their natural brood ball into six-well plates to monitor larval development as described in Shafiei et al. (2001). Larvae were then sexed, staged, weighed, and treated with the JH analog methoprene as described in Moczek and Nijhout (2002a,b). To summarize briefly, approximately 48–24 hr before entering the prepupal stage female larvae were weighed and then treated with methoprene (Supelco^{TM}) dissolved in 100% acetone at a ratio of 1:1000 μl. Each larva was treated once with 10 μl (*O. nigriventris* and *O. binodis*) or 6 μl (*O. sagittarius*) of the solution, applied externally to the ventral side of the highly membranous abdominal cuticle using a micropipette. This treatment achieved an average dosage of approximately 40 μg/g larva in all three species, which was similar to dosages found effective in earlier studies (Emlen and Nijhout, '99, 2001; Moczek and Nijhout, 2002). For unknown reasons *O. nigriventris*, but not *O. binodis* or *O. sagittarius*, suffered severe mortality and additional unwanted side effects due to the use of acetone as a methoprene carrier. We remedied this response by switching to 100% ethanol as a carrier substance for methoprene for *O. nigriventris*, but continued to use acetone for the other two species. Both acetone and ethanol readily permeate the highly membranous ventral cuticle of the larval abdomen. Two control groups were also used. Untreated control females received no application of any kind during their development, whereas solvent-only control animals received control treatments of 100% ethanol (*O. nigriventris*) or acetone (*O. binodis, O. sagittarius*), respectively. Larvae were then returned to their well and permitted to pupate. First to second-day pupae were measured, weighed, and again returned to their well until adult eclosion. Eclosing adults were retained in brood balls for an additional 3–4 days to allow full hardening of the adult cuticle, then weighed, killed, stored in ethanol, and re-measured as described below. Here we focus on pupal measurements, which most accurately reflect prepupal growth, in contrast to adult measurements, which are the outcome of the sum of both prepupal growth and pupal remodeling (Moczek, 2006b). By focusing on pupal measurements we can therefore precisely quantify the effects, if any, of JH on the prepupal growth period of horn development.

Morphometric measurements—horn length

Female pupae of all three species were measured using a two-dimensional image analysis system including a digital video camera (Scion) mounted onto a dissecting scope (Leica MZ 16) and interfaced with a PC using Image J software. Pupal thorax width was used as an estimate for body size (for justification see Emlen, '94; Moczek and Emlen, '99). Pupal pronotal horn length was measured as the linear distance from the anterior-most point of the prothorax, or "tip" of the horn, to the posterior-most point of the prothorax bordering anterior to the scutellum of the second thoracic segment (for justification and discussion of landmarks see Moczek, 2006b). Pupal head horn length in *O. sagittarius* was measured as the linear distance from the tip of the horn to its base on the head surface.

Morphometric measurements—horn shape

As explained above, wild type *O. binodis* pupae are not sexually dimorphic with respect to horn length. However, they are sexually dimorphic with respect to a subtle difference in pupal horn shape (Fig. 2). Male *O. binodis* pupae express a conspicuous medial groove in the pupal horn, which

increases in depth with increasing body size (not shown). We hypothesized that this groove, which is almost entirely absent in female pupae, is involved in generating the vertical ''drop'' seen between the lateral and medial aspects of the horn of large adult males but not females (Fig. 2). We tested this hypothesis by measuring pupal groove depths and adult horn drops of males as they developed from pupae to adults. Each male was thus measured twice, first as a pupae and then again as an adult. Pupal groove depth and adult horn drop were measured as indicated in Figure 2C. We then used linear regressions to describe the average relationship between pupal body size and pupal groove depth, and between adult body size and adult horn drop, respectively. We characterized individuals based on the difference between observed values and the value expected given their pupal or adult size, i.e., their residual pupal groove depth or adult drop depth, respectively. If the pupal groove generates the adult horn drop, residual groove depths and residual drop depths should exhibit a positive correlation, i.e., individuals that express relatively smaller or larger grooves as pupae should also express relatively smaller or larger horn drops as adults. Here, we first show that pupal groove depth indeed determines adult horn drop depth, and then explore the effects of exogenous methoprene on the expression of this sexually dimorphic component of horn shape in female *O. binodis*.

Statistical analyses

We employed a residual analysis to explore the effects of JH treatment on female horn morphology. Horn length measurements from untreated control animals were used to fit a linear regression describing the average relationship between body size and female horn length. This then allowed us to calculate the expected horn length for a given body size in solvent only-treated and methoprene-treated females, which could then be characterized by the difference between the observed and expected horn length given their individual body size, or their *residual* horn length. If methoprene treatment induces horn expression in female *Onthophagus* beetles, residual horn lengths should be positive, whereas inhibition of female horn growth through methoprene should be manifest in negative horn length residuals. If methoprene treatment has no effect on female horn growth, horn length residuals of control and methoprene-treated females should be statistically indistinguishable. We used the same basic approach to analyze the effects of methoprene on the development of a male-like horn groove in methoprene-treated *O. binodis* females. We used groove

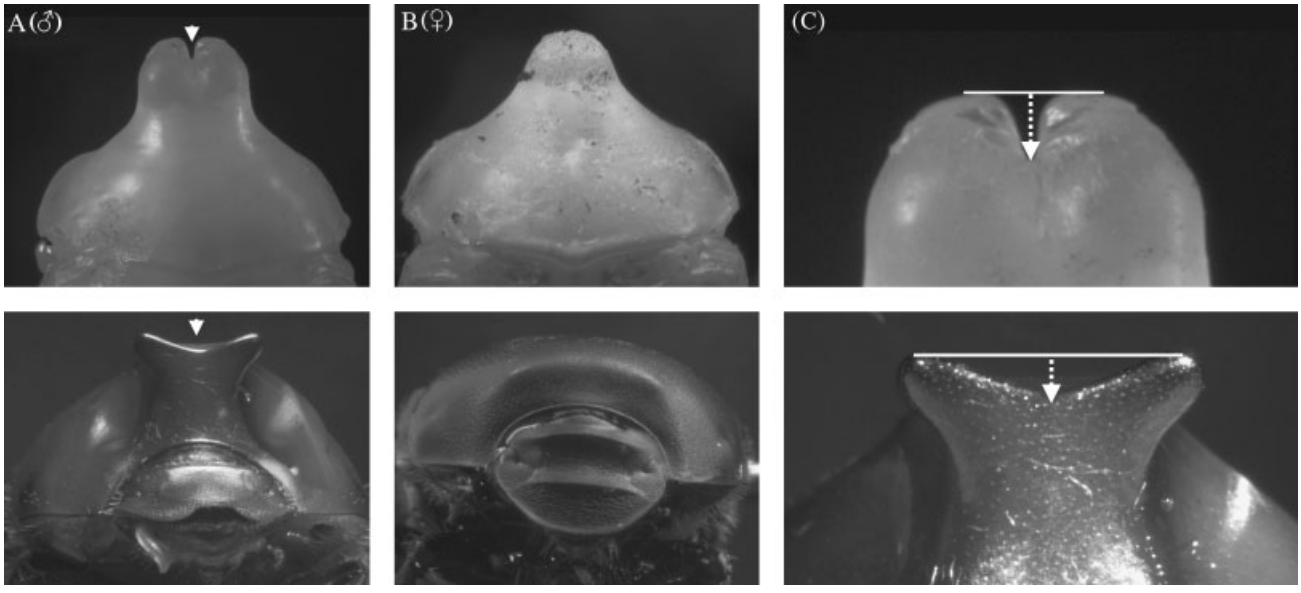

Fig. 2. Sexual dimorphism in pupal and adult horn shape in *Onthophagus binodis*. (**A**) Dorsal view of male pupal prothorax (top) and frontal view of the resulting adult (bottom). Arrows highlight conspicuous medial groove in the horns of male pupae and medial drop in the horns of the resulting adults. (**B**) Corresponding views of female pupal (top) and adult (bottom) prothorax. Wildtype females lack both a pupal horn groove and adult horn drop. (**C**) Detail of the same images shown in (**A**). Arrows indicate how pupal groove depth and adult horn drop were measured.

expression in untreated females to obtain a base line value for female groove expression as a function of female body size, and characterized solvent only-treated females and methoprene-treated females based on the difference between observed and expected groove depths. We further included in this analysis groove depth measurements of male *O. binodis* to evaluate the degree and direction of possible morphological responses detected in females. We used two-tailed *t*-tests for all pair-wise comparisons and employed sequential Bonferroni procedures to correct for multiple comparisons where necessary (Sachs, '92; Sokal and Rolf, '95). Results from *t*-tests are presented as $t_{\text{degrees of freedom}} = \text{test statistic}$. Unless otherwise noted, all results are presented as means $\pm$standard error.

RESULTS

Effect of methoprene on female horn length

In all three species treatment with only solvent had no significant effect on pupal horn length in females (Fig. 3; *O. binodis*: $t_{15} = 1.15$; $P = 0.27$; *O. nigriventris*: $t_{16} = 0.78$; $P = 0.45$; *O. sagittarius* pronotal horn: $t_{36} = 1.71$; $P = 0.1$; *O. sagittarius* head horn: $t_{20} = 1.75$; $P = 0.09$). Treatment with the JH analog methoprene, on the other hand, highly significantly reduced horn length in females of both species with regular sexual dimorphism (Fig. 3; *O. binodis*: $t_{73} = 5.71$; $P < 0.001$; *O. nigriventris*: $t_{32} = 4.91$; $P < 0.001$) but had no consistent effect on pronotal horn expression in *O. sagittarius*, the species with reversed sexual dimorphism (*O. sagittarius* pronotal horn: $t_{30} = 1.70$; $P = 0.23$). Instead,

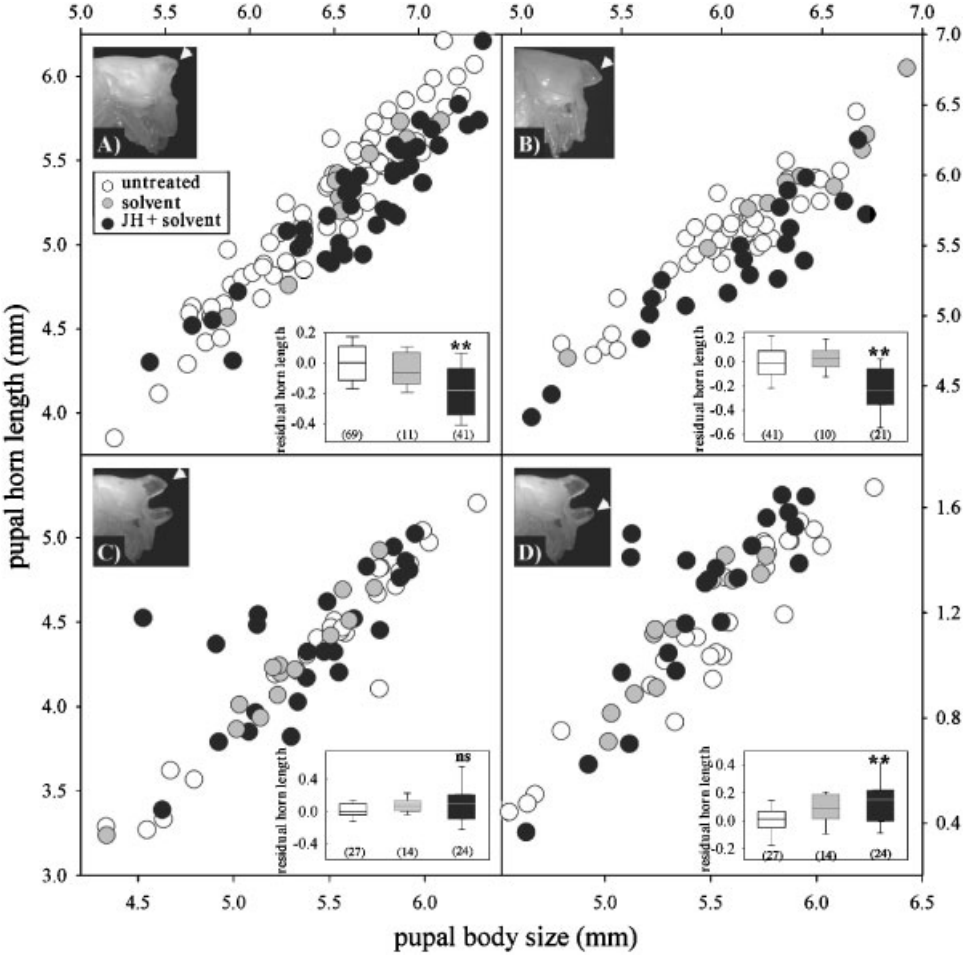

Fig. 3. Scaling relationship between pupal thorax width and horn length for the pronotal horns of female (**A**) *Onthophagus binodis*, (**B**) *O. nigriventris*, (**C**) *O. sagittarius* and (**D**) the head horns of female *Onthophagus sagittarius,* in response to control and methoprene treatment. Sample sizes are indicated in parentheses. Inset: Box plots of residual horn lengths of control and treatment groups. Methoprene treatment reduced prepupal horn growth in *O. binodis* and *O. nigriventris*, had no effect on pronotal horns in *O. sagittarius*, and increased head horn length in *O. sagittarius* ($^{**}P < 0.001$).

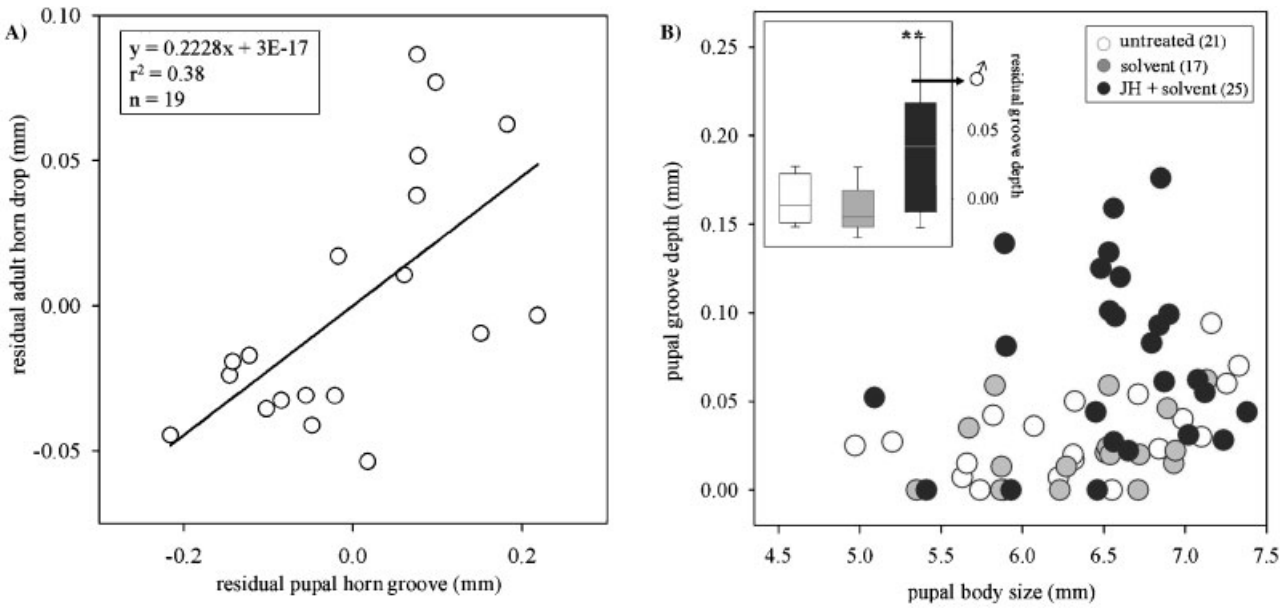

Fig. 4. (**A**) Significant positive correlation between relative (residual) pupal groove depth (*x*-axis) and relative adult horn drop ($P<0.005$) indicates that the male pupal horn groove is a developmental determinant of the adult horn drop. (**B**) Scaling relationship between pupal thorax width and pronotal groove depth in female *O. binodis* in response to control and methoprene treatment. Sample sizes are indicated in parentheses. Inset: residual horn lengths of control and treatment groups. Arrow indicates corresponding mean residual groove depth of male *O. binodis*. Methoprene treatment masculinizes female horn shape by inducing a male-like horn groove in females ($^{**}P<0.001$).

in this species head horn expression responded to methoprene treatment, resulting in significantly enlarged pupal head horns in methoprene treated female *O. sagittarius* compared with control females (*O. sagittarius* head horn: $t_{30} = 1.70$; $P = 0.0029$).

Regulation of horn shape in male O. binodis

Relative pupal groove depth was positively correlated with the depth of the adult horn in *O. binodis,* i.e., males that expressed relatively small (or large) grooves as pupae molted into adults with relatively small (or large) vertical horn drops (Fig. 4A; ANOVA: $F = 10.55$, $P = 0.0047$). The pupal horn groove therefore represents an important morphological precursor of sexual dimorphism in horn shape in adult *O. binodis*. Treatment with only solvent had no significant effect on groove depths in female *O. binodis* pupae (Fig. 4B; $t_{34} = 1.12$; $P = 0.27$). Instead, both untreated and solvent-only treated females expressed only minimal horn grooves. Methoprene treated females, however, exhibited rather dramatic groove expression, in many cases similar to or even exceeding that of their male counterparts (Fig. 4B; $t_{33} = 3.43$; $P<0.005$). Although exposure to methoprene thus feminized *O. binodis* females with respect to horn length, it masculinized them by inducing the expression of a male-like medial horn groove.

DISCUSSION

Sexual dimorphism is a phylogenetically widespread phenomenon, and the evolutionary origin, current maintenance, and developmental basis of sexual dimorphisms are central themes in biology. Here we explore the endocrine regulation of sexual dimorphism in a group of insects that has become a promising model system for studying phenotypic evolution and diversification: horned beetles (reviewed in Emlen, 2000; Moczek, 2005, 2006a,b). Previous studies exploring the regulation of alternative male morphologies in *Onthophagus* beetles have suggested that JH may play an important role in regulating the degree of horn expression in alternative horned and hornless male morphs (Emlen and Nijhout, '99, 2001). In these studies ectopic applications of methoprene to larval *O. taurus* 24–48 hr before the gut purge and thus onset of the prepupal stage induced expression of relatively long head horns in males that normally would remain short-horned,

effectively shifting the threshold body size required for horn induction to smaller body sizes. On the basis of this observation a simple model was constructed, which postulated that male larvae differ in their JH titers depending on their body weight, and that there is a brief sensitive period during late larval development during which JH titers serve as a proxy for future adult body size. Under this model only male larvae heavy enough to express JH titers that exceed a certain titer threshold during this sensitive period will develop into horned males, whereas those exhibiting small body mass, and consequently, titers below the threshold, will become hornless (Emlen and Nijhout, '99). Subsequent work confirmed the existence of this late JH sensitive period and, more importantly, showed that evolutionary changes in degree and timing of sensitivity to JH have provided important avenues for evolutionary changes in patterns of horn expression in geographically isolated populations (Moczek, 2003; Moczek and Nijhout, 2003). Here we show that JH also influences horn expression in females of three *Onthophagus* species. In females of two of the three species studied here, however, ectopic methoprene applications of compatible dosage, and applied at the same time in development compared with earlier studies that focused on males, had the opposite effect with respect to horn length. Rather than inducing horn growth, as observed in males, methoprene significantly reduced the amount of female pronotal horn growth that occurred during the prepupal growth phase. Interestingly, methoprene treatment resulted in increased horn expression in the third species, *O. sagittarius*, but only in one horn type. Although in this species thoracic horn expression was unaffected regardless of treatment, head horns were significantly enlarged in methoprene-treated females compared with control animals. Here, the direction of response to methoprene treatment was similar to what was observed for the head horns of male *O. taurus* in previous studies. Combined, these data suggest that JH may indeed be influencing horn length in female *Onthophagus*, but appears to be able to do so flexibly and dependent on species and horn location.

JH regulates length and shape of horns independently

Our results also suggest an important additional, and previously overlooked role of JH during horn development, namely the regulation of horn shape. Large male *O. binodis* naturally express a conspicuous medial groove in their pronotal horn during pupal development (Fig. 2), and depth of the pupal groove determines the vertical drop between the lateral and medial aspects of the resulting adult horn and thus a conspicuous component of male horn shape (Fig. 4A). Females naturally express no or only minimal grooves as pupae, and the adult prothorax exhibits no medial vertical drop. Females that were exposed to methoprene, however, expressed similar, and in some cases deeper, horn grooves than their male counterparts, suggesting that JH may not only affect horn length but also horn shape. Intriguingly, although in males groove depth increased with body size and horn length, groove induction in methoprene-treated females occurred *despite* an overall reduction in pupal horn length in the same individuals. This suggests that JH may not only regulate length and shape of horns separately, but can do so in opposing directions. If correct, this implies that even though length and shape of horns are both regulated in part by JH, joint regulation is not imposing much of a constraint, and instead allows the same hormone perturbation to have a feminizing effect with respect to horn length, and a masculinizing effect with respect to horn shape. This may be possible if length and shape are determined by different developmental mechanisms. Preliminary results suggest that the medial groove in *O. binodis*, as well as horn shape components in other species, are the product of local changes in epithelial cell shape as well as apoptotic cell death near the end of the prepupal growth phase (T. Kijimoto, J. Andrews, and A. Moczek, unpublished). Horn shape would thus be determined via localized developmental events occurring later in development, utilizing a fundamentally different set of genetic and developmental mechanisms, than horn growth itself. If confirmed, this would help explain how both processes may be able to evolve and diversify independent of one another, whereas at the same time maintaining a joint regulation by circulating JH titers.

The endocrine regulation of horned beetles—what do we really know?

While exploring the role of JH in the regulation of alternative male morphs, Emlen and Nijhout ('99) also explored contributions of another class of hormones, ecdysteroids, to the same process by

quantifying ecdysteroid titers in small males, large males, and females. Ecdysteroids play a critical role in initiating apolysis and thus the onset of the molting cycle, and ecdysteroid titers increased as expected in all three groups as animals neared the larval-pupal molt. However, Emlen and Nijhout ('99) also observed a small ecdysteroid peak several days earlier during the feeding phase of the last larval instar of female larvae and male larvae fated to develop into the small, hornless morphs, but not in males fated to develop into the large, horned morph. Ecdysteroids have been shown repeatedly to play a major role in inducing changes in gene expression in developing tissues. Emphasizing the need for future studies, Emlen and Nijhout ('99) therefore cautiously suggested that the low ecdysteroid titers observed in female and small male larvae may serve to program a hornless morphology in both groups of individuals via a shared endocrine regulatory process. Unfortunately, ecdysteroid titers have never been replicated in this or any other species, and functional tests using ectopic ecdysteroid applications failed to confirm a function of the early ecdysteroid peak in both females and small males (D.J. Emlen, personal communication). In a follow-up study, Emlen and Nijhout (2001) then further explored the role of JH during earlier larval stages around the time of the observed ecdysteroid peak and found that exposure to ectopic methoprene at this stage inhibited rather than induced horn growth of male beetles. Methoprene-treated males developed relatively shorter horns (measured on a qualitative scale from 0 = no horns and 1 = intermediate horns to 2 = large horns) compared with untreated controls. On the basis of this observation, Emlen and Nijhout (2001) argued for a second, earlier sensitive period during which JH titers allow male larvae to decide between two alternative developmental pathways, horned or hornless, whereas JH titers experienced during the originally described, later sensitive period may regulate the exact amount of horn growth that occurs. Unfortunately, the reduction in horn length resulting from methoprene treatment was potentially confounded by a massive delay in the timing of pupation of methoprene treated animals, and thus the amount of time animals were able to gain weight before the pupal molt, and possibly the final adult body size at eclosion. These concerns notwithstanding, subsequent publications (Emlen and Allen, 2004; Emlen et al., 2005a,b) have since combined these observations into a more complex model. This model argues that JH titers present during the feeding stage signal nutritional conditions. Male larvae exposed to poor conditions express reduced JH titers, which trigger a small ecdysteroid peak, programming the larval epidermis to become unresponsive to JH titers experienced just before the prepupal stage, thus inducing a hornless morphology. Male larvae experiencing optimal feeding conditions instead express elevated JH titers and no ecdysteroid peak, and become competent to express horns. Exact amount of horn growth, then, is regulated by JH titers experienced just before the prepupal stage. Lastly, a similar small ecdysteroid peak is thought to be involved in inducing a hornless morphology in females, even though females were thought to be unresponsive to JH manipulations. Interestingly, since the original empirical data generated by Emlen and Nijhout ('99, 2001) and Moczek and Nijhout (2002) summarized above, no additional data have become available, yet the complexity of the endocrine regulation of beetle horns has grown steadily with subsequent publications (Emlen and Allen, 2004; Hartfelder and Emlen, 2005; Emlen et al., 2005a,b; Emlen, 2006). Most importantly, components of this model are now presented as evidence supporting a possible role of endocrine mechanisms in biasing patterns of evolutionary diversification. For example, Emlen et al. (2005a) argued that the shared endocrine regulation of hornlessness in females and small males explains concerted patterns of evolution of alternative male morphs and sexual dimorphism in onthophagine beetles. Here we begin to supply empirical, functional data that may help to re-evaluate this perspective.

We show that female *Onthophagus* indeed respond to similar JH titer manipulations as their male counterparts, at the same time during late larval development, approximately 24–48 hr before the onset of the prepupal stage. Female responses to JH, however, where rather variable, and depended on species, horn region, and horn shape. Our results therefore suggest that even though the endocrine regulation of horn dimorphisms within and between sexes and species share important regulatory components (e.g., the use of JH as a master regulator), we see surprising evolutionary lability within a very narrow phylogenetic framework of exactly how JH influences different aspects of horn expression in different developmental contexts. This suggests that the endocrine regulation of adult morphology in *Onthophagus* beetles is far more developmentally

flexible than previously assumed and, contrary to recent studies, less likely to impose a general macroevolutionary constraint (Emlen et al., 2005a,b). Specifically, these results suggest that females and small males arrive at their largely hornless morphologies via different developmental mechanisms, which is further supported by recent comparative gene expression study on horn patterning genes (Moczek and Nagy, 2005; Moczek et al., 2006). A deeper understanding of the evolutionary endocrinology of horned beetles will, however, critically depend on our ability to gain more quantitative insights into the hormonal regulation of phenotypic diversity in these organisms. For example, a basic question that remains to be addressed is whether JH-mediated changes in horn expression actually result from changes in JH titers or reflect, at least in part, differential sensitivity of target tissues. Therefore, further studies are now under way to finally obtain JH titers as well as titers of the most relevant JH metabolizing enzyme, JH-Esterase, for several *Onthophagus* species (M. Stansbury, T. Taub-Montemayor, M. A. Rankin, and A. P. Moczek, unpublished data), with the ultimate goal to explore the interactions between endocrine and genetic regulators of horn expression in development and evolution of horned beetles.

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