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Developmental mechanisms of threshold evolution in a polyphenic beetle

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SUMMARY Polyphenic development is thought to play a pivotal role in the origin of morphological novelties. However, little is known about how polyphenisms evolve in natural populations, the developmental mechanisms that may mediate such evolution, and the consequences of such modification for patterns of morphological variation. Here we examine the developmental mechanisms of polyphenism evolution in highly divergent natural populations of the dung beetle, *Onthophagus taurus*. Males of this species express two alternative morphologies in response to larval feeding conditions. Favorable conditions cause males to grow larger than a threshold body size and to develop a pair of horns on their heads. Males that encounter relatively poor conditions during larval life do not reach this threshold size and remain hornless. Exotic populations of *O. taurus* have diverged dramatically in body size thresholds in less than 40 years since introduction to new habitats, resulting in the expression of highly divergent and novel horn length–body size scaling relationships in these populations. Here we show that larvae of populations that have evolved a larger threshold body size (1) have to accumulate greater mass to become competent to express the horned morph, (2) require more time to complete the final instar, (3) are less sensitive to the juvenile hormone (JH) analogue methoprene, and (4) exhibit a delay in the sensitive period for methoprene relative to other developmental events. JH has been shown previously to control horn expression in this species. Our results show that threshold evolution may be mediated via changes in the degree and timing of sensitivity to JH and may result in correlated changes in the dynamics and duration of larval development. Strain-specific differences in JH sensitivity have previously been demonstrated in other insects. However, to the best of our knowledge this is the first demonstration that changes in the timing of the sensitive period for JH may play an equally important role in the evolution of novel thresholds. We discuss our findings in the context of the developmental regulatory mechanisms that underlie polyphenic development and use our results to explore the consequences of, and constraints on, polyphenism evolution in nature.

INTRODUCTION

Adaptive phenotypic plasticity can be defined as a genotype's ability to adjust patterns of phenotype expression to suit current or future environmental conditions (Via et al. 1995). Adaptive phenotypic plasticity is carried to extremes in polyphenic organisms. Here, individual genotypes are able to express two or more discrete phenotypes in response to differences in the internal or external environment experienced by the developing organism (Nijhout 1999). Polyphenic development generates a remarkable degree of phenotypic diversity in a wide range of taxa and is thought to play a pivotal role in speciation and the evolution of morphological and behavioral novelties (e.g., protozoans [Kuhlmann and Heckmann 1985; Wicklow 1988], rotifers [Stemberger and Gilbert 1984; Roche 1993], crustaceans [Grant and Bayly 1981; Lively 1986a,b], insects [Shapiro 1976; Koch and Bückmann 1984; West-Eberhard 1989, 1992; Kingsolver 1995; Zera and Denno 1997; Weaver 1957; Wheeler and Nijhout 1983; Crespi 1988; Emlen 1994; Nijhout 1999], chelicerates [Radwan 1993], and plants [Schmalhausen 1949]).

The proximate mechanisms that regulate polyphenic development have been studied on a variety of levels (Nijhout 1999). In general, polyphenic organisms determine which phenotype to develop into based on external factors that reliably indicate future environmental conditions, such as photoperiod (Tauber and Tauber 1970; Koch and Bückmann 1987), temperature (Brakefield and Reitsma 1991; Smith 1978; Hazel and West 1979; Grayson and Edmunds 1989), nutrition (Wheeler 1986; Denno et al. 1986; Greene 1989, 1996; Emlen 1994; Moczek 1998), crowding (Denno et al. 1986; Harris 1987; Zera and Tiebel 1989), presence or absence of predators (Stemberger and Gilbert 1984; Lively 1986a,b; Pfennig 1990), or a combination of the above (Hardie and

Lees 1985; Wheeler and Nijhout 1983, 1984). Differences in external conditions are then translated into differences in internal parameters, often involving endocrine or neuroendocrine factors (Okkot-Kotber 1980; Wheeler and Nijhout 1983; Endo and Funatsu 1985; Hardie 1987; Koch and Bückmann 1987; Wheeler 1991; Zera and Tobe 1990; Pener 1991; Tanaka and Pener 1994; Rountree and Nijhout 1995; Zera and Denno 1997; Emlen and Nijhout 1999; Starnecker and Hazel 1999). By means of response thresholds, continuous variation in internal conditions is used to elicit discrete developmental responses: above-threshold conditions cause an individual to follow a particular developmental pathway, leading to the expression of a particular phenotype, whereas below-threshold conditions cause an individual to take an alternative pathway, leading to the expression of a discretely different alternative phenotype (Nijhout 1994, 1999). Geographic comparisons and breeding experiments have demonstrated that response thresholds can vary heritably among populations, suggesting that threshold evolution may provide an important avenue for phenotypic diversification in polyphenic taxa (Tauber and Tauber 1972, 1982; Harrison 1979; Hazel and West 1982; Semlitsch and Wilbur 1989; Semlitsch et al. 1990; Zera et al. 1996; Denno et al. 1996; Emlen 1996, 2000; Roff 1996; Ahlroth et al. 1999). Furthermore, several studies have provided an initial insight into how developmental mechanisms may need to be modified to result in novel threshold responses (Zera and Tiebel 1989; Zera and Tobe 1990; Gu and Zera 1995; Zera and Zhang 1995; Roff et al. 1997). However, insights from natural populations remain scarce, and the developmental mechanisms that actually mediate threshold evolution in nature are largely unknown.

Here we examine the developmental mechanisms of threshold evolution in highly divergent natural populations of the polyphenic dung beetle *Onthophagus taurus*. Male *O. taurus* vary continuously in body size in response to differences in larval feeding conditions (Moczek 1998; Moczek and Emlen 1999). Males above a critical threshold body size express a pair of disproportionally long curved horns on their heads, whereas smaller males develop only rudimentary horns or no horns at all (Fig. 1). As a consequence of this threshold, natural populations of *O. taurus* are composed of two discrete male morphs, and intermediate morphologies are rare (Moczek and Emlen 1999). *O. taurus* originally exhibited a circum-Mediterranean distribution (Balthasar 1964). In the early 1970s *O. taurus* was introduced accidentally to the eastern United States and, as part of a biocontrol program, to Western Australia (Fincher and Woodruff 1975; Tyndale-Biscoe 1996). Since introduction to their new habitats, both exotic populations have undergone rapid evolutionary divergence in the critical body size threshold that separates alternative morphs (Moczek 2002; Moczek et al. 2002). The threshold divergence of US and Australian populations is of a kind and magnitude similar to differences between species within the genus and

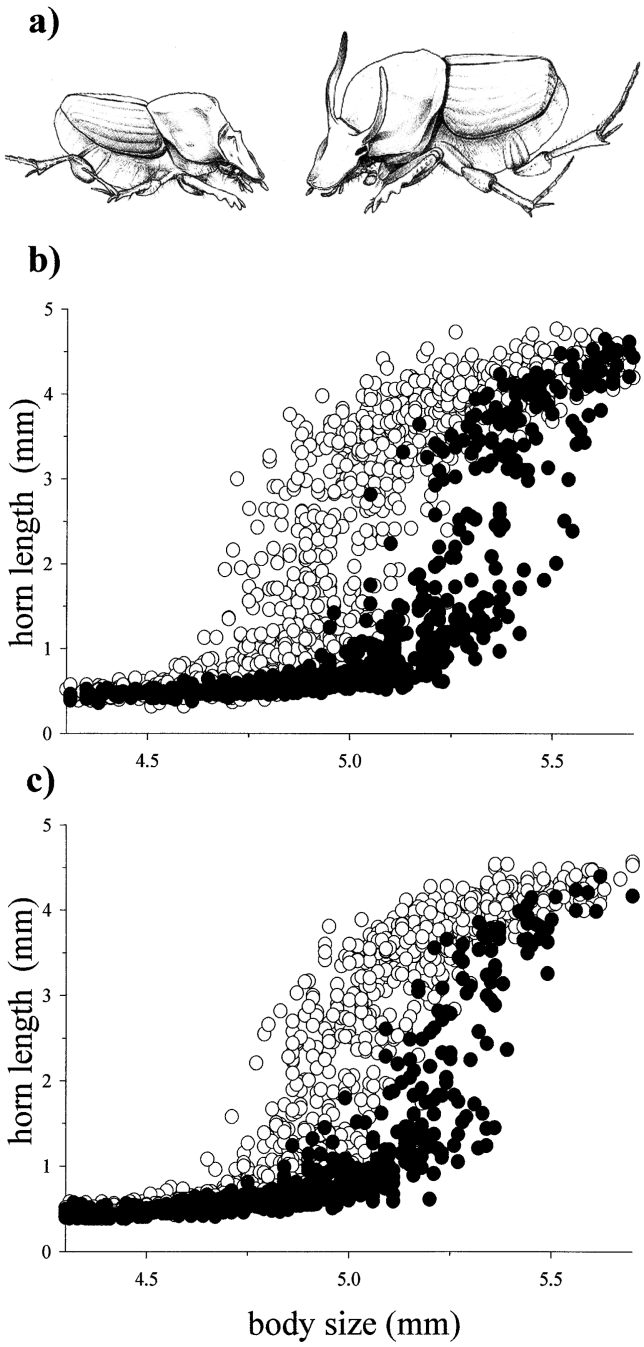


Fig. 1. Male horn polyphenism in exotic populations of *Onthophagus taurus*. (A) Typical hornless and horned male morphology. (B) Scaling relationship between body size and horn length of field collected *O. taurus* (open dots: North Carolina, $n = 1019$; solid dots: Western Australia, $n = 644$). (C) Scaling relationship between body size and horn length of laboratory-reared *O. taurus* at the time at which the experiments described here were carried out (open dots: North Carolina, $n = 938$; solid dots: Western Australia, $n = 784$).

persists in the laboratory under a common garden rearing regime (Fig. 1) (see also Moczek et al. 2002).

Earlier studies demonstrated that juvenile hormone (JH) plays a crucial role in the determination of male horn phenotype (Emlen and Nijhout 1999, 2001). Topical application of the JH analogue methoprene can induce horn expression in small males destined to express the hornless male morph, provided application occurs during a 24- to 48-h window before the gut purge and the onset of the prepupal stage (Emlen and Nijhout 1999). Based on these findings, Emlen and Nijhout (1999) developed a model of the endocrine control of horn expression, which suggests that male larvae differ in

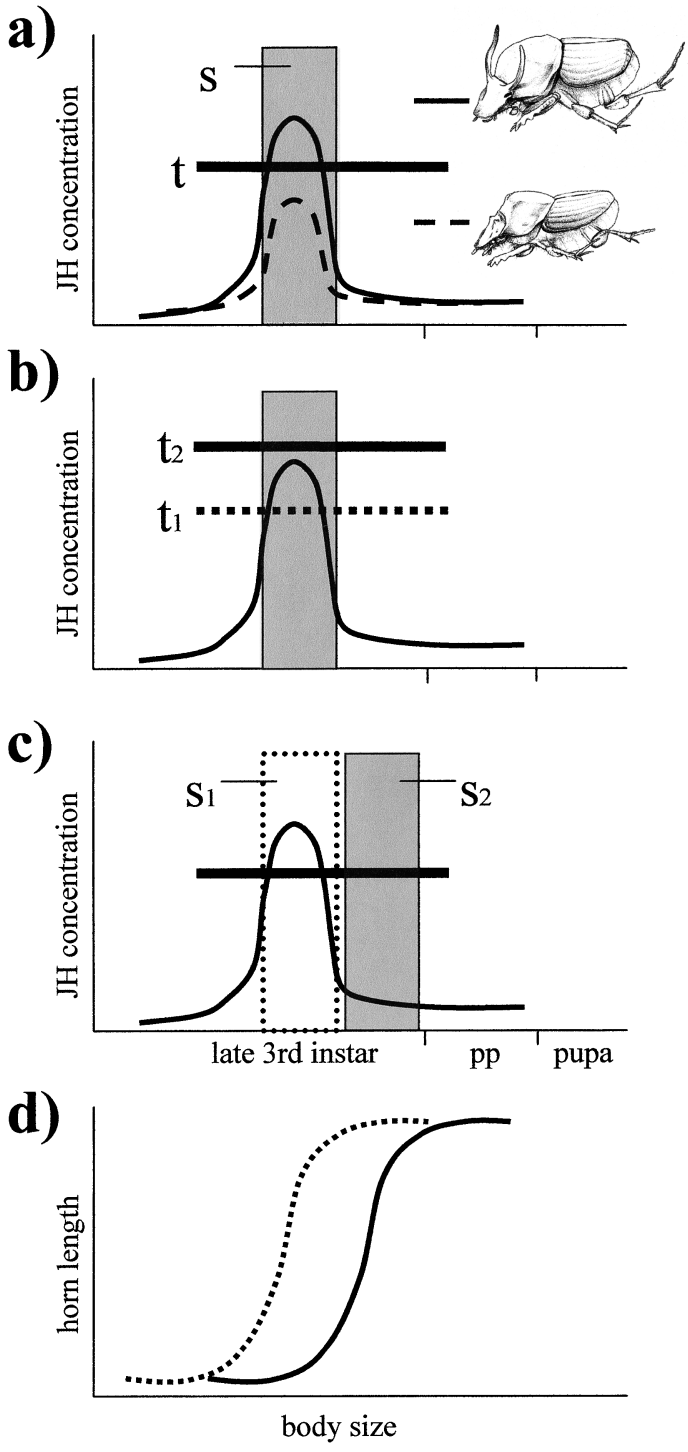


Fig. 2. Endocrine control of male horn dimorphism and potential developmental mechanisms that mediate threshold evolution in exotic populations of *Onthophagus taurus.* (A) Endocrine control of male horn dimorphism (after Emlen and Nijhout 1999). Males are assumed to differ in juvenile hormone (JH) titers depending on their body size. Only large males express JH titers above a threshold (**t**) during a certain sensitive period (**s**) and will develop horns as adults, whereas smaller males with JH titers below the threshold will remain hornless. (B) Elevation in the JH threshold ($\mathbf{t}_1$ to $\mathbf{t}_2$) causes medium-sized male larvae to express JH titers below the threshold necessary for horn development and to now express the hornless instead of horned morph as adults. (C) A delay in the JH-sensitive period ($\mathbf{s}_1$ to $\mathbf{s}_2$) relative to JH secretion results in JH titers of medium-sized male larvae to fall below the JH threshold necessary for horn development before the horn-developing tissue acquires JH sensitivity, causing these males to now express the hornless instead of horned morph as adults (pp = prepupal stage). (D) On the level of a population both developmental modifications (B and C) would result in a shift of the critical threshold body size to larger body sizes.

their JH titers depending on their body mass. According to the model, small male larvae exhibit JH titers below a certain threshold concentration during a well-defined sensitive period and consequently develop into the hornless morph. Larger male larvae express JH titers above this threshold and develop into the horned morph (Fig. 2A) (Emlen and Nijhout 1999). This model suggests at least two major developmental avenues for threshold evolution. First, changes in the sensitivity to JH could alter the location of the body size threshold (Fig. 2B). Reduced sensitivity, for example, would cause males that would have expressed JH titers just above the threshold to now develop into the hornless, instead of the horned, morph (Fig. 2B). Second, changes in the timing of sensitivity to JH relative to the temporal pattern of JH secretion could also result in a modification of the body size threshold (Fig. 2C). For example, if the JH sensitive period normally occurs during a high but falling phase of JH titers, then a delay in the sensitive period could now cause it to coincide with JH titers that fall below the threshold required to induce horn growth. As a consequence, males who previously expressed JH titers just above the threshold now fall below the threshold and consequently will express the hornless male morph. At the level of a population, both mechanisms would be manifest as a shift of the body size threshold to larger body sizes (Fig. 2D).

Here we explore the developmental mechanisms underlying the rapid evolution of divergent body size thresholds in exotic *O. taurus* populations. We begin by contrasting the dynamics of larval development and the relative timing of developmental events during the final instar of larvae derived from North Carolinian and Western Australian *O. taurus* populations. We then examine how North Carolinian and Western Australian *O. taurus* differ in their sensitivity to the JH analogue methoprene and the timing of this sensitive period relative to other developmental events.

MATERIAL AND METHODS

Origin of strains and rearing protocol

For our study we used two laboratory strains derived from about 2000 beetles collected near Busselton, Western Australia (WA) and 1500 beetles collected near Durham, North Carolina (NC), respectively. In both areas *O. taurus* inhabits pasturelands with largely similar climatic conditions. Colonies were past the third generation in captivity at the start of the experiment. Both laboratory strains were kept in the same insectary at Duke University at 26°C and 60% relative humidity under a 16:8 light:dark cycle. Under these conditions both strains were found to breed readily and without obvious differences in fecundity (Moczek, unpublished data). Beetles were bred in plastic containers (25 cm tall, 20 cm Ø) filled three fourths with a moist sand–soil mixture. Five pairs of beetles were added to each container (eight containers per colony per week) and provided with approximately 0.5 liters of homogenized cow dung. Six days later beetles were removed and brood balls were collected. By this time most larvae had already hatched and passed through the first instar. The following day, a subset of brood balls was carefully opened, and second-instar larvae were transferred into artificial growth containers and provided with an unlimited food supply for the remainder of the larval stage (for details on containers and diet, see Shafiei et al. 2001). Larval growth containers were kept in a constant temperature room at 26°C, 60% relative humidity, and in complete darkness except for a brief daily examination. The remaining brood balls were used to rear beetles for colony maintenance. To minimize inbreeding, individual adult beetles were allowed to produce brood balls only once and were then removed from the colony. Different generations were kept in separate containers. Over 1000 individuals were reared each generation for each strain. Great care was given to provide both laboratory strains with the exact same treatment and breeding set up.

Quantifying larval growth trajectories and the timing of developmental events

Larvae in artificial growth containers were observed daily. Once larvae molted to the third (final) instar (scored by head capsule size; Moczek and Nijhout, unpublished data), larvae were weighed daily to the nearest 0.001 g using a high sensitivity balance (Mettler AE50, Mettler-Toledo Inc., Columbus, OH). We also recorded the day on which genital imaginal disks first became visible (males only; see Moczek and Nijhout 2002 in press), the day on which larvae reached their peak mass, the day on which larvae purged their gut and entered the prepupal stage, and the days on which individuals pupated and eclosed as adults. We used these developmental markers to subdivide the third larval instar into four clearly recognizable stages: molt into third instar to first appearance of genital imaginal disks (stage 1), first appearance of genital imaginal disks to peak mass (stage 2), peak mass to end of gut purge (stage 3), and end of gut purge to pupation (prepupal stage; stage 4). Because only male larvae expressed visible genital imaginal disks, stages 1 and 2 had to be combined into a single stage for female larvae. We also measured horn length and thorax width of eclosing adults (see below).

Degree and timing of sensitivity to JH

Synchronization of larval development

A subset of larvae reared using the above protocol was used to quantify the degree and timing of sensitivity of NC and WA third instar *O. taurus* larvae to the JH analogue methoprene. Our method for quantifying JH sensitivity at particular developmental stages (see below) required that all experimental and control animals follow similar developmental trajectories. To synchronize developmental trajectories of larval *O. taurus*, we used recent insights into the proximate mechanisms that control the onset of metamorphosis in onthophagine beetles. In nature, *O. taurus* larvae appear to use food cessation as a cue for the initiation of metamorphosis (Shafiei et al. 2001). Experimental removal of *O. taurus* larvae from their food source causes larvae to undergo a stereotypic and highly predictable sequence of developmental transitions, eventually leading to pupation. Removing individuals from their food source at the same growth stage effectively synchronizes their subsequent development (Shafiei et al. 2001). To obtain larvae of similar developmental trajectories, we therefore removed larvae from their food

source on day 10 of the third instar by transferring larvae into growth containers that contained a moist sand–soil mixture but no manure. At this stage, most larvae had already reached their peak mass and had accumulated enough reserves to complete metamorphosis and to express the full range of body sizes and horn lengths found in natural populations. Food removal at this time is likely to correspond to the point at which larvae would have consumed most of their brood ball under natural circumstances. In our experiment, removing larvae from their food source drastically reduced variation between individuals in the timing of subsequent developmental events and caused the large majority of larvae to pupate within a 24-h interval 7 days after food removal (Moczek and Nijhout, unpublished data).

Experimental approach

Changes in sensitivity to JH and changes in the timing of the sensitive period for JH may account for the expression of divergent threshold body size in NC and WA populations (Fig. 2). We used topical applications of the JH analogue methoprene to artificially induce horn expression in male larvae fated to express the hornless morph (see below). We used the proportion of larvae that expressed horns after methoprene treatment as an estimate of a strain's mean sensitivity to a given methoprene concentration applied at a given point during late larval development. To test whether strains differed in their sensitivity to JH, we treated larvae with one of three different methoprene concentrations. Methoprene was dissolved in acetone to obtain concentrations of 0.2, 1, and 5 μg/μl, and 10 μl of one of these solutions was applied topically to the ventral surface of a larva using a micropipette. Using this method we achieved approximate dosages of 20, 100, and 500 μg/g larva, respectively. The highest concentration (500 μg/g larva) is similar to the concentration used by Emlen and Nijhout (1999). Control animals were treated with an equal volume of pure acetone. To test whether NC and WA larvae differed in the timing of the sensitive period for JH, we applied methoprene 3, 4, 5, and 6 days after larvae were removed from their food source (= days 13, 14, 15, and 16 of the third instar, respectively). Because preliminary data indicated that WA, but not NC larvae, exhibit a delayed sensitive period for JH, we also treated WA larvae on day 7 after food removal (= day 17 of the third instar).

Selection of animals

Hormone manipulation experiments required larvae that normally would metamorphose into male adults expressing the hornless phenotype. We determined sex of developing larvae by scoring for the presence of visible genital imaginal disks. Disks are expressed only in males as early as day 4 of the third larval instar and provide a reliable and noninvasive way for sexing *O. taurus* in the larval stage (Moczek and Nijhout 2002 in press). We estimated the prospective horn phenotype of incipient male larvae based on larval body mass attained by day 10 of the third instar. Larval mass accumulated by this time explains over 80% of adult size variation ($r^2 = 0.82$, $n = 27$). To express horns as adults, larval mass has to exceed at least 0.1500 g by day 10 of the third instar before larvae were removed from their food source. Larvae weighing less than 0.1500 g on day 10 before food removal developed into hornless males without exception ($n = 49$; Moczek and Nijhout, unpublished data). Therefore, only male larvae weighing less than 0.1500 g on day 10 of the third instar were used in the hormone manipulation experiment.

Scoring of horn phenotype

We determined the actual horn phenotype expressed by experimental and control (acetone treated) animals once individuals had reached the pupal stage and horns became clearly visible. Pupae remained in growth containers until their eclosion into adults, which were then stored in ethanol for morphometric measurements. Methoprene applications severely reduced larval ability to complete ecdysis, that is, to completely shed the larval skin during the larval–pupal molt, and all methoprene-treated animals eventually died in the pupal stage. However, because horns evaginate to form the pupal horn as soon as the animal sheds the anterior portion of the larval cuticle, horn phenotype could be scored clearly and unambiguously in all animals even if pupae did not survive to adulthood (see also Emlen and Nijhout 1999). Similar effects of JH analogues on ecdysis have been documented in other insects (e.g., Hatakoshi et al. 1988, Kremen and Nijhout 1989) and are a common side effect of treatment with JH analogues.

Morphometric measurements of adult beetles

All morphometric measurements were taken using a standard two-dimensional image analysis system and Image software (Image Software Inc., West Chester, PA) at the Duke University Morphometrics Laboratory (for details see Moczek 1998, Moczek and Emlen 1999). Horn length was measured as described in Moczek (1998). Thorax width was used as an estimate for body size (for justification see Emlen 1994; Moczek and Emlen 1999).

Statistical analyses

Durations of developmental stages were not normally distributed in many cases. We therefore used multiple nonparametric Mann-Whitney U tests to compare durations of developmental stages between strains. Unless otherwise noted, we report significance values after correcting for multiple comparisons using sequential Bonferroni correction procedures (Sachs 1992; Sokal and Rohlf 1995).

RESULTS

Larval growth dynamics

Differences within strains

In both strains, larvae destined to develop into the horned morph required significantly more time to complete stages 1 and 2 of the third instar than their prospective hornless counterparts ($P < 0.05$ for each comparison). Within-strain comparisons of every other developmental stage indicated no significant differences between morphs with one exception. WA (but not NC) horned males required 1–2 days longer to complete the pupal stage than their hornless counterparts ($P < 0.05$).

In an earlier study on *O. taurus* derived from a WA population, Hunt and Simmons (1997) found that male larvae destined to develop into the horned morph required significantly more time to complete larval development than female larvae that eclosed to adults of similar body sizes. Hunt and Simmons (1997) interpreted their results as a reflection of developmental costs associated with the expression of an exaggerated male secondary sexual trait. Surprisingly, our results failed to support this hypothesis. In our experiment

horned males of either strain did not show a significant difference or even tendency to require more time to complete the third larval instar, prepupal, or pupal stage than their similar-sized female counterparts.

Differences between strains

NC and WA individuals differed significantly in the peak mass that male larvae had to reach to develop into the horned morph (Fig. 3). NC larvae whose peak mass exceeded 0.14 g regularly expressed horns as adults, whereas WA larvae had to exceed nearly 0.16 g to metamorphose into the horned morph (Fig. 3).

WA larvae also required significantly more time to complete the third instar than NC larvae, independent of whether larvae developed into horned males, hornless males, or females (Fig. 4; $P < 0.01$ for each comparison). Using developmental markers to further subdivide the third instar, we found that male larvae did not differ in the duration of stage 1, which ends with the first appearance of visible genital imaginal disks (Fig. 4, A and B). However, male WA larvae required significantly more time to reach their peak mass and the prepupal stage (stages 2 and 3, respectively) compared with their NC counterparts (Fig. 4). Differences between strains were significant, independent of whether larvae subsequently developed into horned or hornless males ($P < 0.05$ for each comparison). WA larvae that developed into female adults also required significantly more time to reach their peak mass ($P < 0.05$) and the prepupal stage ($P = 0.023$; not significant after correction for multiple comparisons) than their NC counterparts. We found no significant differences between strains in the duration of the prepupal (stage 4) or pupal stage (Fig. 4).

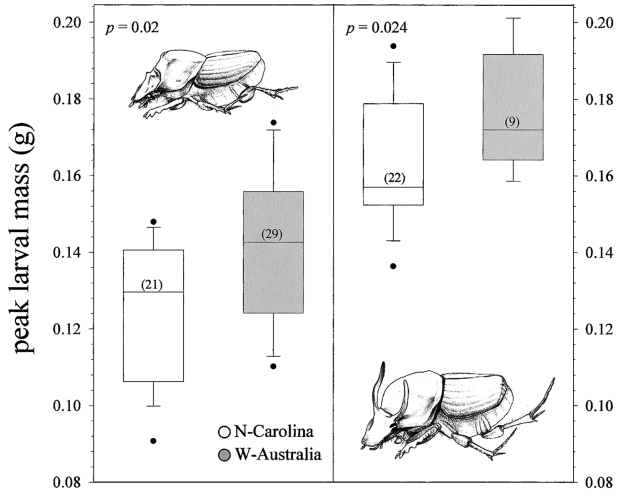


Fig. 3. Peak mass of *Onthophagus taurus* larvae as a function of male morph and strain (left: hornless males; right: horned males; white boxes: North Carolina; gray boxes: Western Australia). Larvae had ad libitum access to food. Western Australian male larvae had to accumulate a significantly higher peak mass to become competent to express horns as adults than their North Carolinian counterparts (Mann-Whitney U test; sample sizes are given in parentheses).

Sensitivity to methoprene and its timing

NC larvae treated with acetone never expressed horns (Fig. 5; $n = 25$). Likewise, larvae treated with the lowest methoprene concentration never switched developmental pathways and expressed horns regardless of when methoprene was applied. Instead, larvae developed into hornless pupae in all cases ($n = 26$). NC larvae treated with medium or high methoprene concentrations on day 3 after food removal (= day 13 of the third instar) never pupated ($n = 12$) and instead remained inactive for up to 2 weeks and eventually died. NC larvae treated with medium methoprene concentrations switched developmental pathways and expressed horns in 40% of the cases when methoprene was applied on day 4 ($n = 15$) and in 16.7% of the cases when methoprene was applied on day 5 ($n = 12$) but in no case when methoprene was applied on day 6 post food removal (n = 9; Fig. 5). Similarly, NC larvae treated with high methoprene concentrations expressed horns in 47% of the cases when methoprene was applied on day 4 ($n = 19$) and in 40% of the cases where methoprene was applied on day 5 ($n = 10$) but again in no case when methoprene was applied on day 6 after food removal ($n = 11$; Fig. 5). Results for the highest methoprene concentration were consistent with earlier findings by Emlen and Nijhout (1999).

WA larvae likewise never expressed horns in response to acetone treatment ($n = 27$) or application of the lowest methoprene concentration ($n = 27$) regardless of when it was applied (Fig. 5). Similarly, WA larvae treated with medium or high methoprene concentrations on day 3 after food removal never successfully pupated and instead remained inactive until they starved to death in all cases ($n = 8$). However, unlike NC larvae, WA larvae failed to respond to medium methoprene concentrations at any developmental stage and instead remained hornless in all cases (Fig. 5; $n = 28$). WA larvae did however respond to the highest methoprene concentration, but only when treated on day 6 after food removal (43%, $n = 14$). Larvae treated with the highest methoprene concentration on any other developmental stage remained hornless in all cases ($n = 26$). Combined, these results indicate that incipient hornless male larvae of both strains differ not only in their sensitivity to the JH analogue methoprene, but also in the timing of the sensitive period relative to other developmental events.

DISCUSSION

The significance of polyphenic development in the evolution of phenotypic diversity has received much attention (Schmal-

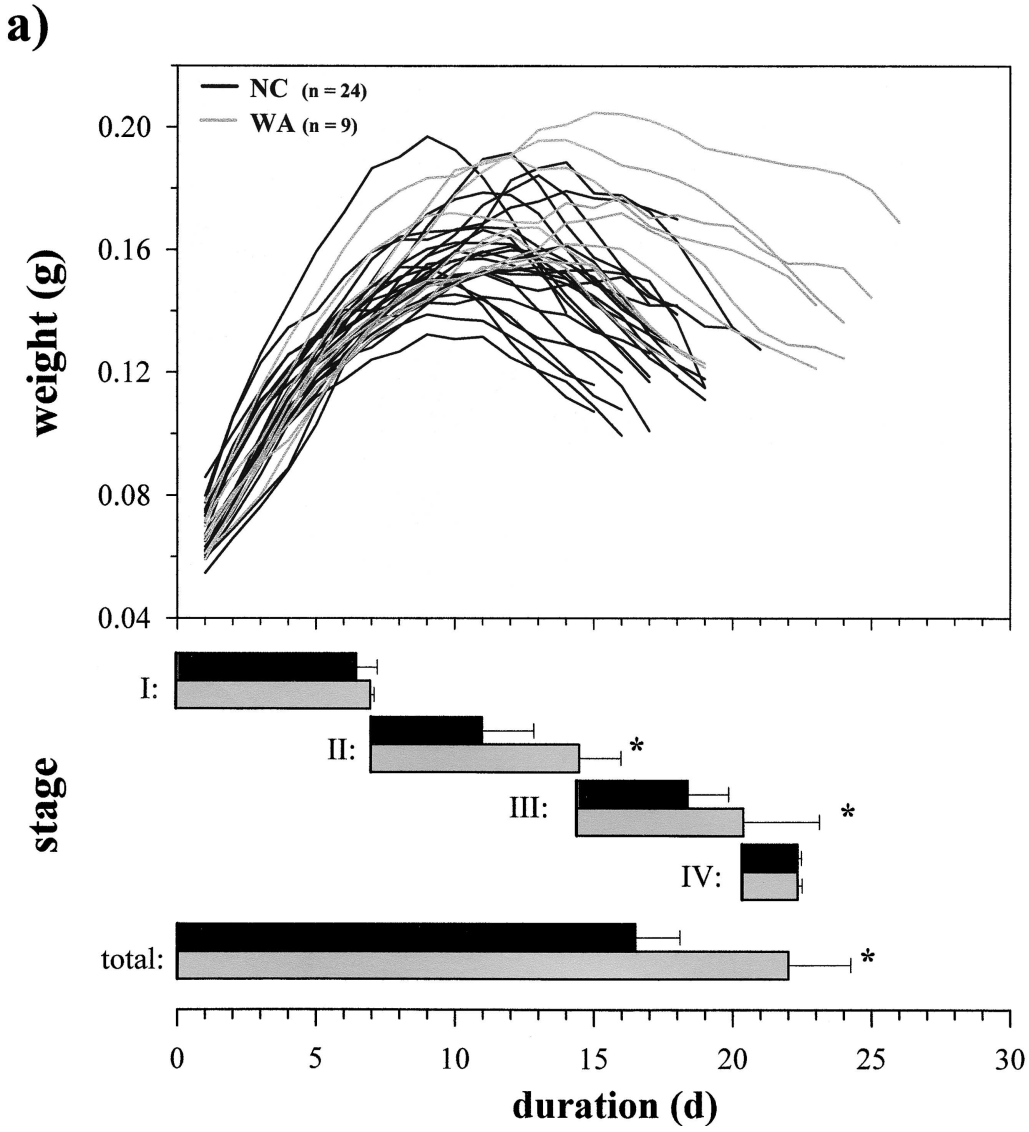
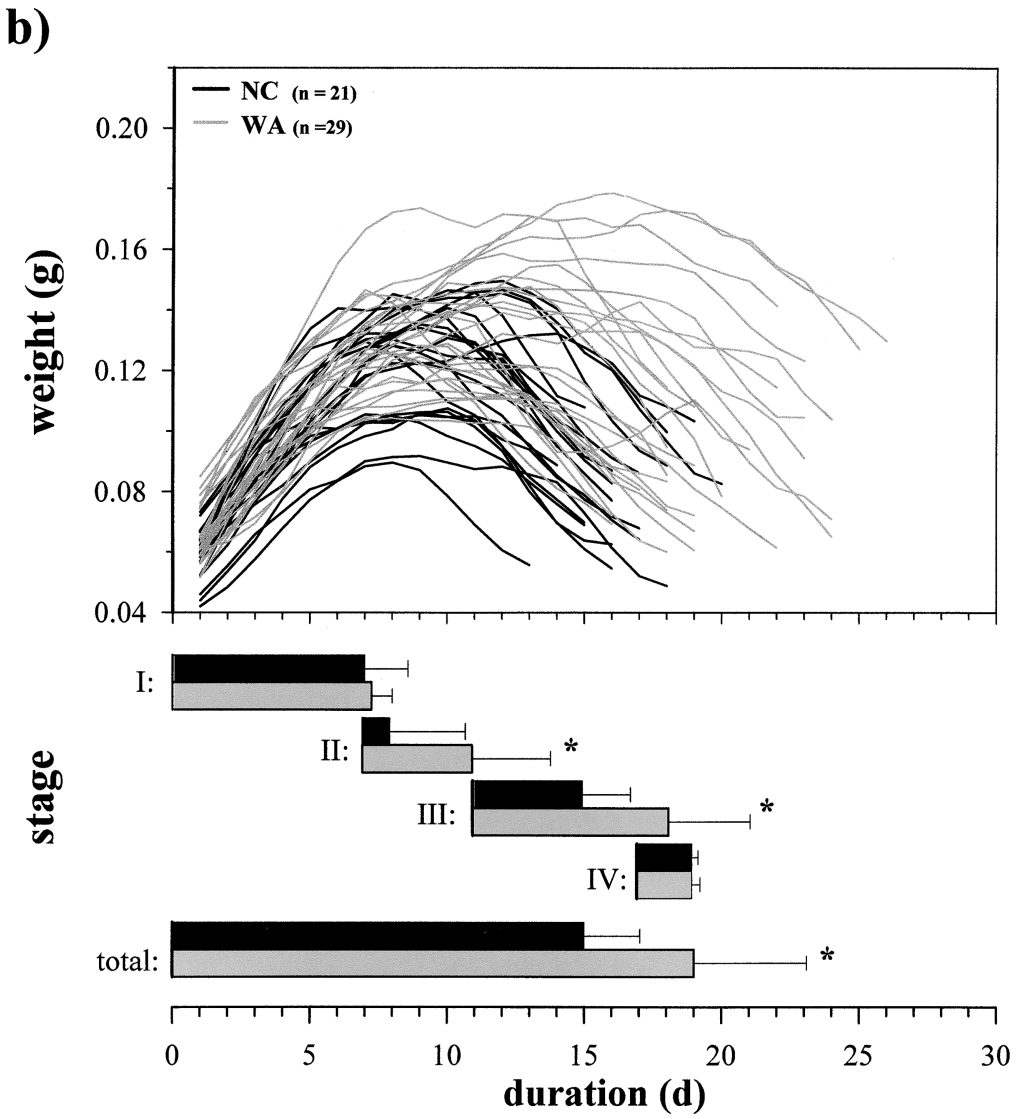


Fig. 4. Dynamics and duration of larval development in *Onthophagus taurus.* Growth curves and stage durations of third instar (A) presumptive horned males, (B) presumptive hornless males, and (C) female larvae. Larvae had ad libitum access to food (gray lines and bars: Western Australia; black lines and bars: North Carolina; stage I: molt to third instar to first appearance of genital imaginal disks; stage II: first appearance of genital imaginal disks to peak mass; stage III: peak mass to gut purge; stage IV: prepupal stage; $*P < 0.05$; Mann-Whitney U tests including sequential Bonferroni correction procedures for multiple comparisons). Stages I through IV are lined up according to their mean time of onset for the Western Australian strain. With exception of the first and last day of the instar, growth curves are presented as three-point moving averages. Results indicate that Western Australian larvae require significantly longer to reach peak mass and to transition to the prepupal stage, resulting in a significant extension of the third larval instar and delayed pupation.

hausen 1949; West-Eberhard 1989, 1992; Brakefield et al. 1996; Schlichting and Pigliucci 1998). However, surprisingly little is known about how polyphenisms evolve in natural populations, the developmental mechanisms that may mediate such evolution, and the consequences of such modification for patterns of morphological variation. We previously showed that exotic populations of the polyphenic dung beetle *O. taurus* evolved highly divergent body size thresholds in less than 40 years since introduction to new habitats (Moczek 2002; Moczek et al. 2002). Here we show that divergent threshold responses may be achieved developmentally by subtle changes in the sensitivity to JH and by modification of the timing of the sensitive period for JH during late larval development. Strain-specific differences in the sensitivity to JH were previously suggested to be responsible for differences in morph expression patterns in hemipterans



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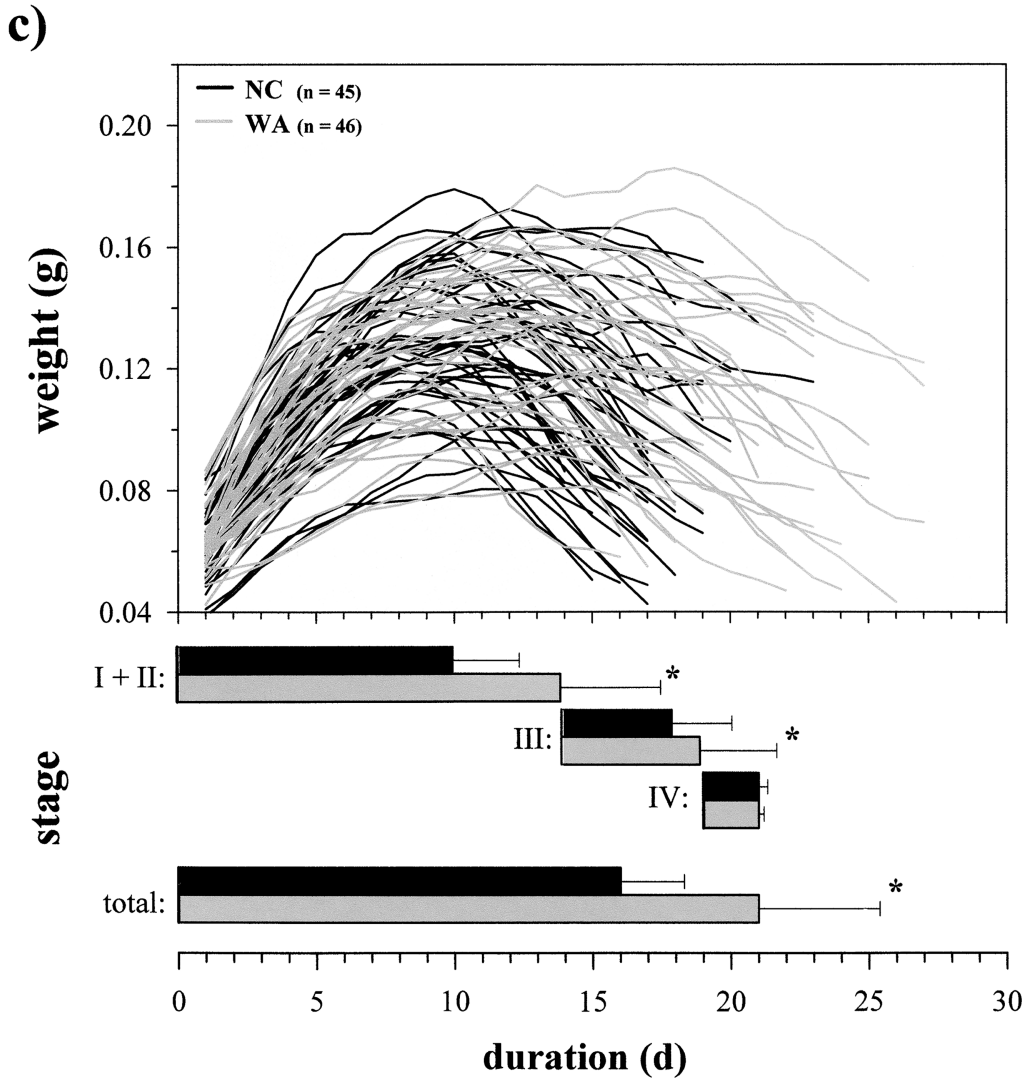
(Dingle and Winchell 1997), suggesting that evolutionary modification of JH sensitivity may be a common mechanism that mediates the evolution of novel response thresholds in insects. In the present work we demonstrate that shifts in the timing of a sensitive period for JH may play an equally important role in the evolution of novel thresholds.

Our results suggest that evolutionary modifications of response thresholds in *O. taurus* can have consequences for the duration and dynamics of larval development as a whole. Male WA *O. taurus* had to accumulate significantly higher mass than their NC counterparts to become competent to express the horned morph. Furthermore, WA individuals required significantly more time to reach their peak mass and to transition to the prepupal stage, resulting in a substantial increase in the duration of the final larval instar. Strain-specific differences in the duration of larval development were unexpected and could have evolved for reasons unrelated to the evolution of novel response thresholds. Alternatively, strain-specific differences in the duration of larval development may be direct consequences of modified response thresholds and may therefore provide important insights into the consequences of, and constraints on, threshold evolution in onthophagine beetles. JH is involved in the regulation of numerous larval developmental events and plays a central role in the coordination of molting, pupation, and metamorphosis (Nijhout 1994, 1999). Pupation generally requires the *absence* of JH during a particular sensitive period during late larval development (Nijhout 1994, 1999). In *O. taurus* this latter period is preceded by the sensitive period for horn induction. Here, JH has to be *present* above a certain concen-



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tration to induce horn expression (Emlen and Nijhout 1999; this study). A delay in the sensitive period for JH-mediated horn expression, as is the case in WA *O. taurus*, may cause a correlated delay in subsequent JH-sensitive periods, such as the one involved in regulating pupation, which in turn would result in an extension of the larval stage. If this hypothesis is correct, delayed pupation and an extended larval stage would reflect correlated responses to an evolutionary modification of the threshold response that mediates horn expression. Interestingly, a delay in pupation is also seen in hornless males and female WA *O. taurus*, even though neither express horns. This indicates that although the evolutionary alteration of the developmental threshold for horns has changed the morphology of only large males, the underlying developmental modifications required to achieve this alteration may have had consequences for all other members of the population.

If delayed pupation and the following extension of the larval stage are indeed a consequence of the evolution of the threshold, this could place severe constraints on threshold evolution in onthophagine beetles. Metabolic limitations, such as those arising from a limited pool of resources available to developing larvae (Moczek 1998; Shafiei et al. 2001), probably place an upper limit on how long pupation can be delayed and consequently the extent to which threshold evolution can be mediated by delaying the timing of the sensitive period. Furthermore, Hunt and Simmons (1997) showed that *O. taurus* larvae that require more time to complete the larval stage are more likely to be attacked by nematodes; hence, extending larval development may also carry with it increased

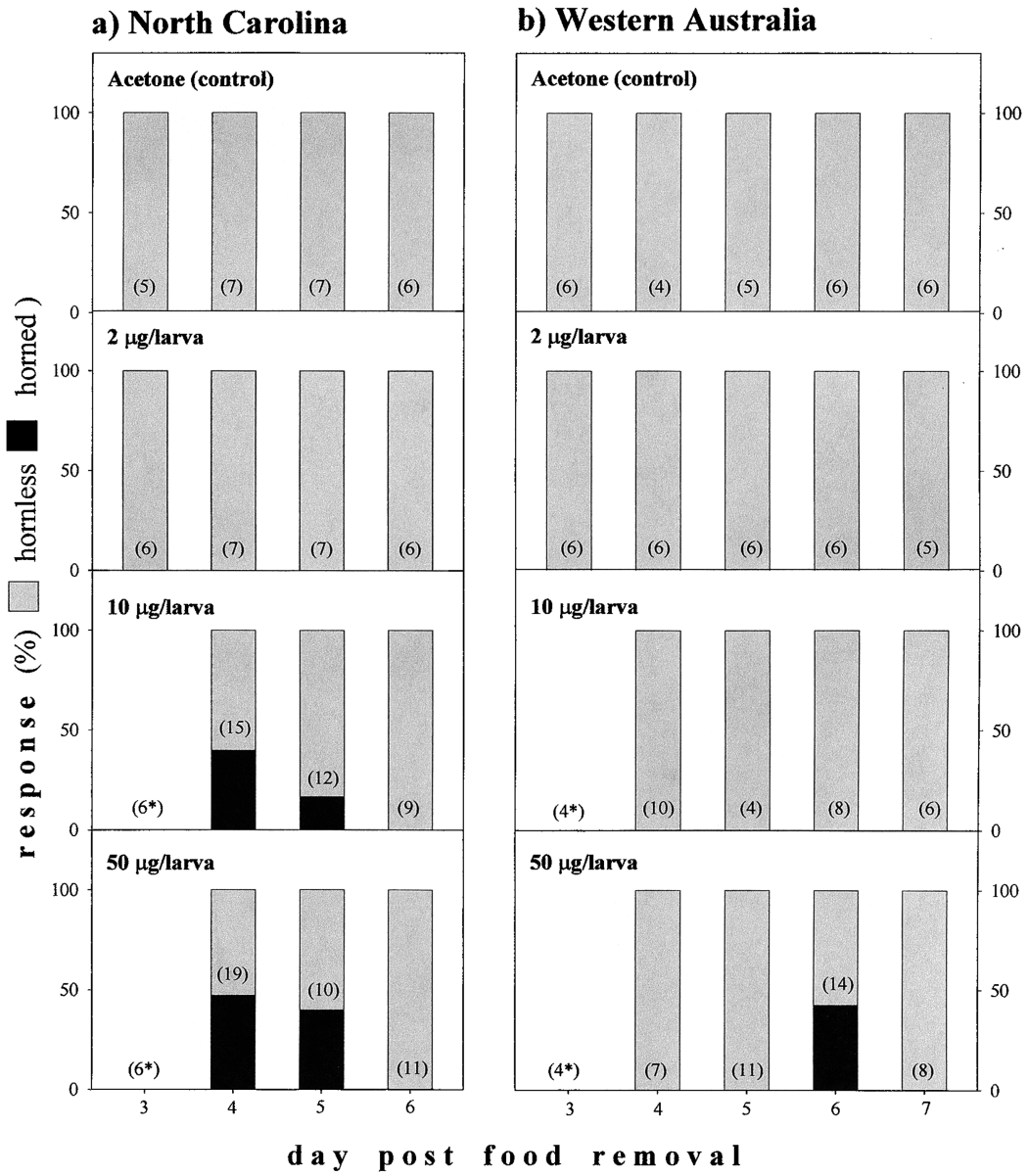

Fig. 5. Juvenile hormone (JH) sensitivity and timing of the sensitive period for JH in incipient hornless male *Onthophagus taurus* larvae from (A) North Carolina and (B) Western Australia. To synchronize larval development, larvae were removed from their food source at day 10 of the third instar. Larvae were treated with a single dose of the JH analogue methoprene 3, 4, 5, and 6 days after larvae were removed from their food source. Western Australian larvae were also treated on day 7 after food removal. Three different methoprene dosages were used: 2, 10, and 50 μg/larva. X axis indicates the day (after food removal) at which methoprene was applied. Bar height indicates the percentage of males that responded to the treatment and expressed horns (black bars) or did not respond to the treatment and expressed the hornless morph (gray bars). Sample sizes are given in parentheses. *No larvae pupated successfully. Control individuals were treated with an equal volume of pure acetone. Results suggest that both strains differ not only in larval sensitivity to JH but also when this sensitivity is expressed during larval development. See text for further details.

predation risks. Opportunity costs, especially in populations with a short active season and limited resource availability, may further limit the extent to which threshold evolution via a delay in the sensitive period for JH and an extension of the larval stage becomes evolutionarily favorable.

Shifting the body size threshold to *smaller* body sizes by shifting the sensitive period to *earlier* parts of the larval stage is probably also constrained by many factors. Male horn polyphenism plays an important role in male–male competition for females, and earlier studies suggested that the reproductive

success of a male depends on his ability to express a particular optimal horn length relative to its adult body size (Moczek and Emlen 1999, 2000). However, because body size is largely determined by larval feeding conditions, larvae may not be able to predict their future adult body size until fairly late in larval development (Moczek 1998; Shafiei et al. 2001). Shifting the sensitive period for horn expression to earlier parts of the larval stage may therefore detract from the accuracy with which developing male larvae can match horn expression to their future adult body size. Furthermore, shifting the sensitive period for horn expression to earlier parts of the larval stage may interfere with other JH-mediated developmental events that take place during this time.

The genus *Onthophagus*, with over 2000 species worldwide, represents one of the most speciose genera in the animal kingdom (Balthasar 1964). Male horn polymorphism is common in the genus, and many species express very similar horned and hornless male phenotypes and horn length–body size allometries. However, congeners often differ distinctly in the exact location of the body size threshold (Emlen 1996), a pattern also observed in other beetle taxa (e.g., Kawano 1995). Earlier studies therefore suggested that changes in threshold responses may provide an important avenue for phenotypic diversification in this genus (Emlen 1996; Moczek and Emlen 1999). Our results confirm this notion and provide a first insight into the developmental mechanisms that may mediate threshold evolution in natural populations. Furthermore, our results indicate that relatively simple and subtle modifications of the developmental machinery that controls male morph expression may suffice to generate rather substantial differences in response thresholds and resulting adult scaling relationships between body size and horn length. Because allometric differences between NC and WA populations have evolved in less than 40 years after introduction to new habitats, our results also indicate that such developmental modifications can evolve extraordinarily rapidly in geographically isolated populations and may precede, rather than follow, the evolution of reproductive isolation. An important implication of our findings is therefore that allometric diversification may be relatively easy to achieve evolutionarily, provided appropriate driving forces are in place. We are now focusing on identifying which evolutionary forces have caused NC and WA populations, and perhaps other species before them, to diverge in their allometries.

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