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The Evolution and Development of Novel Traits, or How Beetles Got Their Horns

ARMIN P. MOCZEK

How do major novel traits originate and diversify in natural populations? This question addresses one of the most fundamental, yet unresolved, issues in evolutionary biology. Over the past decade, a peculiar group of organisms, horned beetles, has emerged as a model system for understanding the ecological, developmental, and genetic mechanisms that operate during the early stages of innovation and diversification. Here I review this body of research and highlight surprising insights into the interplay between proximate and ultimate mechanisms in the origins of diversity in these organisms.

Keywords: allometry, threshold traits, evolution of novelty, Distal-less, horned beetle

Over the past century and a half, evolutionary biologists have developed several theoretical frameworks for understanding the process of evolution and the underlying forces that power it. Darwin, for example, emphasized the role of adaptation in optimizing shape and behavior of organisms so they can best utilize their environment (Darwin 1872). Others view present-day organisms primarily as the lucky survivors of extinction events, which often randomly wiped out major branches of the evolutionary tree regardless of how well their representatives were adapted to their environment (Gould 1989). Still others view the evolution of organismal shape and behavior, or what biologists call the phenotype, as being governed by physical, biochemical, and developmental rules and limits (Goodwin 1994, Kauffman 1995). According to this view, what type of diversity is allowed to evolve is to a large degree determined by what developmental biology permits. If developmental processes cannot produce a certain variant or novel phenotype, it simply will not evolve, regardless of how well adapted and superior it might have been. Clearly, these alternative viewpoints are not mutually exclusive, and there is now ample evidence that all three perspectives capture important aspects of the evolutionary history of life on Earth (Raff 1996). Where each of them continues to fail, however, is in addressing a fundamental and largely unresolved question in evolutionary biology: How do major novel traits actually originate and diversify in nature?

What are major novel traits, and why are existing theories insufficient to explain their origin? When evolutionary biologists discuss the origin of major novel traits, they typically think about the origins of important structures such as legs,

eyes, and wings, structures whose invention opened up a new dimension of life and made a new sphere of niches available for organisms to occupy. An adaptationist's perspective is useful here, as it helps in understanding how the same structure—say, the leg of an insect—took on different shapes in different groups of insects (such as the walking leg of a ground beetle, the digging leg of a mole cricket, or the predatory grasping leg of a praying mantis): each type of leg is best adapted to the respective ecological conditions of its bearer. But where appendages such as legs came from in the first place, and how and why a lineage evolved that first appendage from an appendage-less ancestor, is typically beyond what an adaptationist's perspective can address. Here, developmental approaches have provided important insights, in particular with respect to the kinds of developmental modifications that are associated with major evolutionary divergences (Brakefield et al. 1996, True and Carroll 2002). However, as valuable as these insights are, they largely inform scientists only about the end product of long periods of divergence between lineages, after a wide range of genetic and developmental modifications were allowed to take place in separate lineages since their initial separation. They do not inform us about how and why such divergences were initiated in the first place. The central, unresolved question thus becomes, What does it take,

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ecologically, developmentally, and genetically, for a major novel trait to originate and diversify? In this article I summarize the first promising results of one such attempt to explore the early stages of the origin and diversification of a novel trait, using a peculiar group of insects that many biologists consider to be morphologically one of the most spectacular and diverse: horned beetles.

Beetle horns: Unique and diverse

Beetle horns combine several characteristics that make them outstanding models for studying the origin and diversification of novel traits.

First, beetle horns are unique structures. While we know that, for example, the wings of bees and butterflies are homologous structures (that is, they represent evolutionary modifications of the same original, ancestral structure), beetle horns lack obvious homologues in other groups of insects. Thus, horns in beetles can be viewed as an evolutionary novelty that beetles obtained at some point during their evolutionary history (Moczek 2005).

Second, beetle horns are major traits. Beetle horns are massive, solid, three-dimensional outgrowths that often severely transform the shape of their bearer (figure 1; Arrow 1951, Mizunuma 1999). Horns are routinely as long as or longer than other appendages such as legs, and in some cases can double the length of an individual and make up more than 30% of its body mass. These structures define their bearers morphologically (and also behaviorally, as we will see later) in many ways, and clearly set them apart from other organisms (Emlen 2001).

Third, beetle horns are tremendously diverse. Several thousand species in at least five beetle families develop horns or horn-like structures of some kind (figures 1, 2; Arrow 1951, Matthews 1972). For example, different species grow horns in different body regions. Some species grow horns from the head, others grow them from the thorax, and still others have multiple horns growing from different regions. Within species, males and females typically differ drastically in horn growth, with females almost always growing no or greatly reduced horns. Even within the male sex of a given species, variation in horn growth can be as extreme as it is between species. In hundreds of species, only large males grow a full set of horns, whereas males smaller than a certain body size threshold develop greatly reduced horns or none at all (Arrow 1951, Matthews 1972). Because the size and shape of adult beetles do not change with age, these differences between males persist through their entire adult lifetime.

This diversity in horn growth among extremely closely related individuals or species provides an unprecedented toolbox to identify genetic and developmental mechanisms that generate variation in horn growth between individuals, as well as the ecological and behavioral causes that ultimately underlie this variation. Previous work that attempted to understand why certain new traits originated and diversified in some organisms but not in others had to rely primarily on comparisons between distantly related taxa that showed ma-

jor differences in phenotype expression. Such taxa, however, typically also exhibited markedly disparate ecologies, which made it difficult to pinpoint the ecological, but also developmental and genetic, conditions present during their early morphological differentiation and diversification. Horned beetles, even though immensely variable on a morphological level, all share many aspects of the same basic developmental biology and ecology, and thus promise significant insight into the early stages of innovation and diversification in nature. But what is the biology of horned beetles, and for what, if anything, do they use their horns?

Fifty ways to beat your rival

Several hypotheses have been suggested to explain the evolution and potential use of beetle horns (Arrow 1951). Horns have been thought to allow beetles to defend themselves against predators, to indicate male quality to choosy females, or to facilitate digging through soil. Alternatively, Arrow (1951) suggested that horns may actually have no function and may simply be the product of selection toward larger body size. More recently, William G. Eberhard, of the University of Costa Rica, presented substantial evidence that beetles in a range of families use their horns primarily as weapons in male-male combat over access to females (Eberhard 1978, Eberhard et al. 2000), a hypothesis that has since been confirmed in a wide range of species (Siva-Jothy 1987, Cook 1990, Emlen 1997, Moczek and Emlen 2000, Hunt and Simmons 2002, Moczek 2005).

In many species, fights occur inside tunnels. Such fights generally involve shoving contests, and horns appear to function primarily to position rival males for powerful blows delivered by upward jerks of the head or thorax (Palmer 1978, Emlen 1997, Moczek and Emlen 2000). In other species, fights occur aboveground, on the soil surface or up in trees. In these cases, horns commonly function to grasp and throw opponents, which can inflict serious (and at times fatal) injuries, such as cracks to the exoskeleton (Siva-Jothy 1987).

Importantly, horns not only are *used* in fights but also significantly boost performance in male-male combat. For example, *Onthophagus taurus* males fight in tunnels underneath dung pads, and large males almost invariably defeat smaller males in direct encounters. Fights between males of similar body sizes, however, are often decided by the relative length of the contestants' horns, and males with relatively larger horns typically prevail in aggressive encounters with smaller-horned but otherwise equally sized opponents (Moczek and Emlen 2000). In fact, even very small differences in horn length can be decisive in these fights, and can result in measurable differences in fertilization success (Hunt and Simmons 2001). Males that engage in fights should therefore benefit from growing the largest horns possible to maximize their chances of winning. Why is it, then, that in most species studied so far, only large males grow horns, whereas smaller males remain hornless throughout their lives?

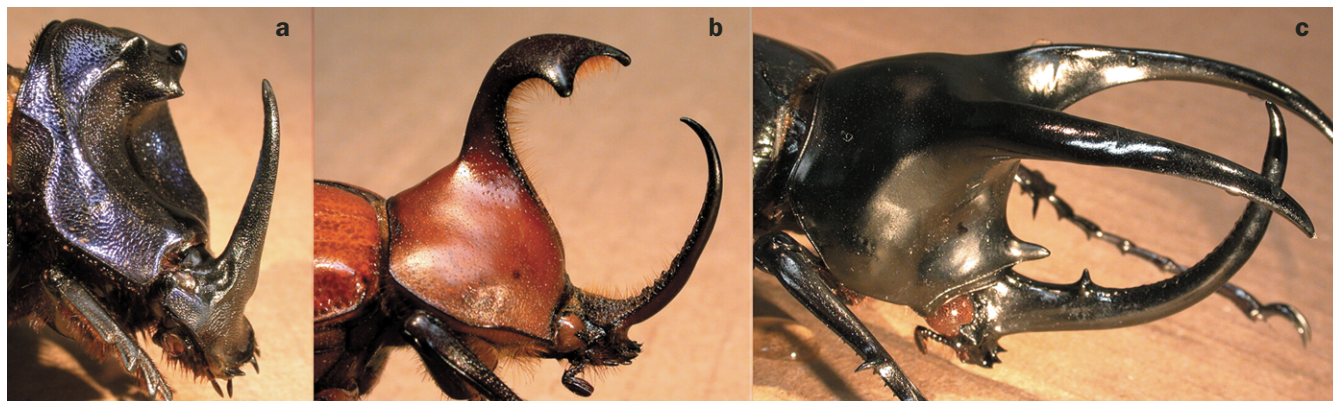


Figure 1. Three examples of horned beetles, illustrating the diversity and magnitude of horn expression in adult beetles. From left to right: *Coprophanaeus ensifer*, *Golofa claviger*, *Chalcosoma atlas*.

One more way to beat your rival

In horn-dimorphic species, small, hornless males commonly do not engage in prolonged fights with large, horned males and instead engage in various types of nonaggressive sneaking behavior (figure 3). Sneaking behavior includes gaining access to breeding tunnels underneath guarding males by using naturally occurring tunnel intersections, digging horizontal interception tunnels, or waiting near tunnel entrances for the guarding male to leave temporarily to assist with food provisioning. In some cases, sneaker males attempt to mate aboveground with females that emerge from breeding tunnels to collect dung for brood ball provisioning (figure 3; Moczek and Emlen 2000).

In two *Onthophagus* species, lack of horns has been shown to significantly enhance the agility of hornless males inside tunnels, which in turn is likely to enhance hornless males' ability to bypass guarding males and locate and mate with breeding females (Moczek and Emlen 2000). Although guarding males may be able to successfully exclude a single sneaker male from entering a breeding tunnel, they are eventually overrun by a group of challengers, allowing at least one sneaker male to gain access to and mate with the female (Hunt and Simmons 2001). Combined, the observational and experimental evidence available to date therefore suggests strongly that horns are indeed advantageous in the context of male–male competition. It also suggests, however, that fighting and horn possession represent only one of at least two solutions to the challenge of gaining access to females in the presence of competing males, and that sneaking and the corresponding lack of horns provide a widespread and nonaggressive alternative tactic.

But why is it that males within a species are of variable size to begin with? And how is it possible that in many species there is a sharp transition from hornless to fully horned individuals, with a paucity of intermediates? Since the size and shape of an adult beetle are final—that is, they do not change during the beetle's remaining lifetime—something prior to the adult stage must therefore influence the size and degree of horn development of individual males.

Where's my food, Mommy?

Horned beetles are most speciose and morphologically diverse in the family Scarabaeidae. Larval development in this family relies predominantly on three different feeding modes: saprophagy, or feeding on decomposing plant matter (e.g., by larvae of *Chalcosoma* species; figure 1c); necrophagy, or feeding on carrion (e.g., by larvae of *Coprophanaeus ensifer*; figure 1a); and coprophagy, or feeding on fecal material (e.g., by larvae of all *Onthophagus* species; figure 2). To allow their offspring access to these resources, adult females lay eggs either in decaying logs, in processed and buried pieces of carrion, or in brood balls made of dung and buried underground.

As different as these resources are, they all share one ecological quality with crucial consequences for larval development: they are finite. A developing larva is limited to the dung ball, carrion ball, or rotting log that its mother selected for it. Once this resource is consumed, horned beetle larvae typically have no opportunity to find other resources to extend larval development. Although mothers presumably do their best to provision their offspring with the resources they need to complete larval development, factors such as climate conditions, humidity, or nutritional quality of the food source are often beyond their control. Therefore, some larvae inevitably find themselves without enough food to reach the larval weight that would allow them to metamorphose into a large adult. In anthropomorphic terms, their choice now is to die as larvae, or to make the best of what they have and metamorphose at a small larval size and into a small adult.

Since larvae appear not to “know” when they run out of food until they actually do, the timing of metamorphosis has to be highly flexible, and not tied to a critical weight as in most other insects. In *O. taurus*, for example, larvae will initiate pupation reliably as soon as their food source is removed (Shafiei et al. 2001). Larvae are capable of doing so after just 5 days of feeding during the final instar, whereas well-fed animals would feed continuously for approximately 16 days before pupating. Starved animals can pupate and metamorphose at less than 40% of the weight at which well-fed individuals would metamorphose, generating a size range among

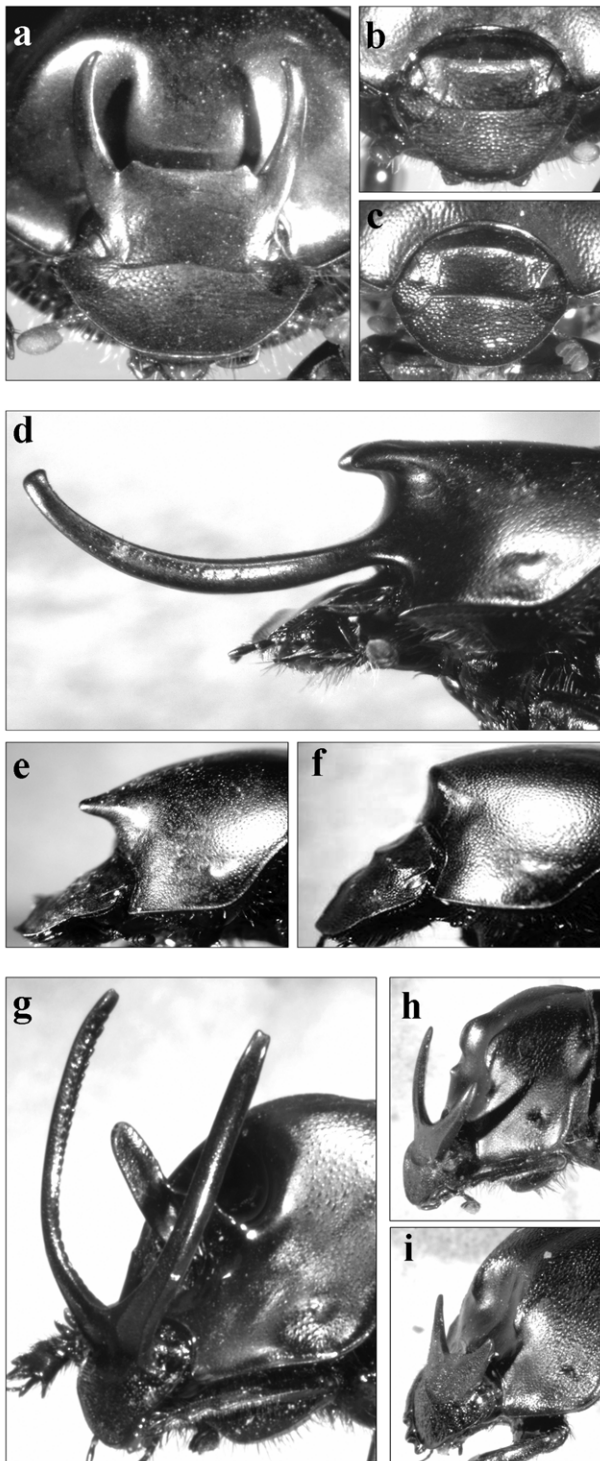


Figure 2. Three types of sexual and intramale diversity in the genus *Onthophagus*. Top: Paired head horns in *Onthophagus australis* ([a] large male, [b] small male, [c] female). Center: Medial thoracic horn in *Onthophagus nigriventris* ([d] large male, [e] small male, [f] female). Bottom: Paired head horns and medial thoracic horn in *Onthophagus watanabei* ([g] large male, [h] small male, [i] female). Note presence of relatively small head horns but complete absence of thoracic horn in (h).

adults that exceeds 100% in both body length and weight (Shafiei et al. 2001). Thus, differences in adult body size are not a function of genetic differences between individuals, but reflect disparate larval feeding conditions, and individuals make do with whatever body size their larval environment allows them to attain (Emlen 1994).

For males that compete with rivals over access to females, this means choosing a behavior that maximizes their chances of success, given their size and thus their strength. As noted above, for large males the decision goes in favor of aggressive fighting behavior and the development of horns as weapons, whereas small males rely on nonaggressive sneaking behaviors, using a more agile, weaponless morphology. But which morphology and behavior is best for a given body size? What determines, for example, the body size threshold below which males should remain hornless and sneak, but above which they should grow the longest horns possible and fight?

To grow or not to grow?

The answers to the questions above have both an ultimate, ecological and a proximate, developmental component. As separate as they may seem at first, we will see shortly how both are intimately intertwined in the diversification of horned beetles. From an ecological perspective, a given male should develop horns only if the reproductive success, or fitness, gained through engaging in fights exceeds the fitness gained through engaging in sneaking behavior, since horns help only in fights, but are useless or even detrimental for sneaking males (Emlen 1997). Since fighting success is heavily determined by body size, it is not surprising that horn expression, too, should scale tightly with body size. Males smaller than some critical body size threshold are too weak to succeed in most fights even if they had horns, and thus are better off engaging in sneaking behavior and not developing horns as adults. Males above this size threshold, however, should have the strength it takes to succeed in most fights, and hence should grow horns to be even more effective fighters. In combination, this is expected to result in an S-shaped (sigmoid) scaling relationship, or allometry, between body size and horn length, where the point of inflection of the S corresponds to the optimal body size threshold between alternative male morphologies and associated reproductive behaviors (Moczek 2003). Such sigmoid allometries between horn length and body size turn out to be very common among horned beetle species, and are particularly widespread in the genus *Onthophagus* (figure 4).

However, at exactly what body size fighting behavior and horn possession become profitable should depend at least in part on external conditions. Any ecological factor that alters the composition of males in a population, or changes the nature of interactions between males, should also affect the body size threshold above which horn expression is favorable. Recent studies on *O. taurus* illustrate that external conditions indeed determine threshold locations and, what is even more important, that changes in these conditions can lead to the evolution of novel threshold sizes and divergences between populations in an extraordinarily rapid fashion.

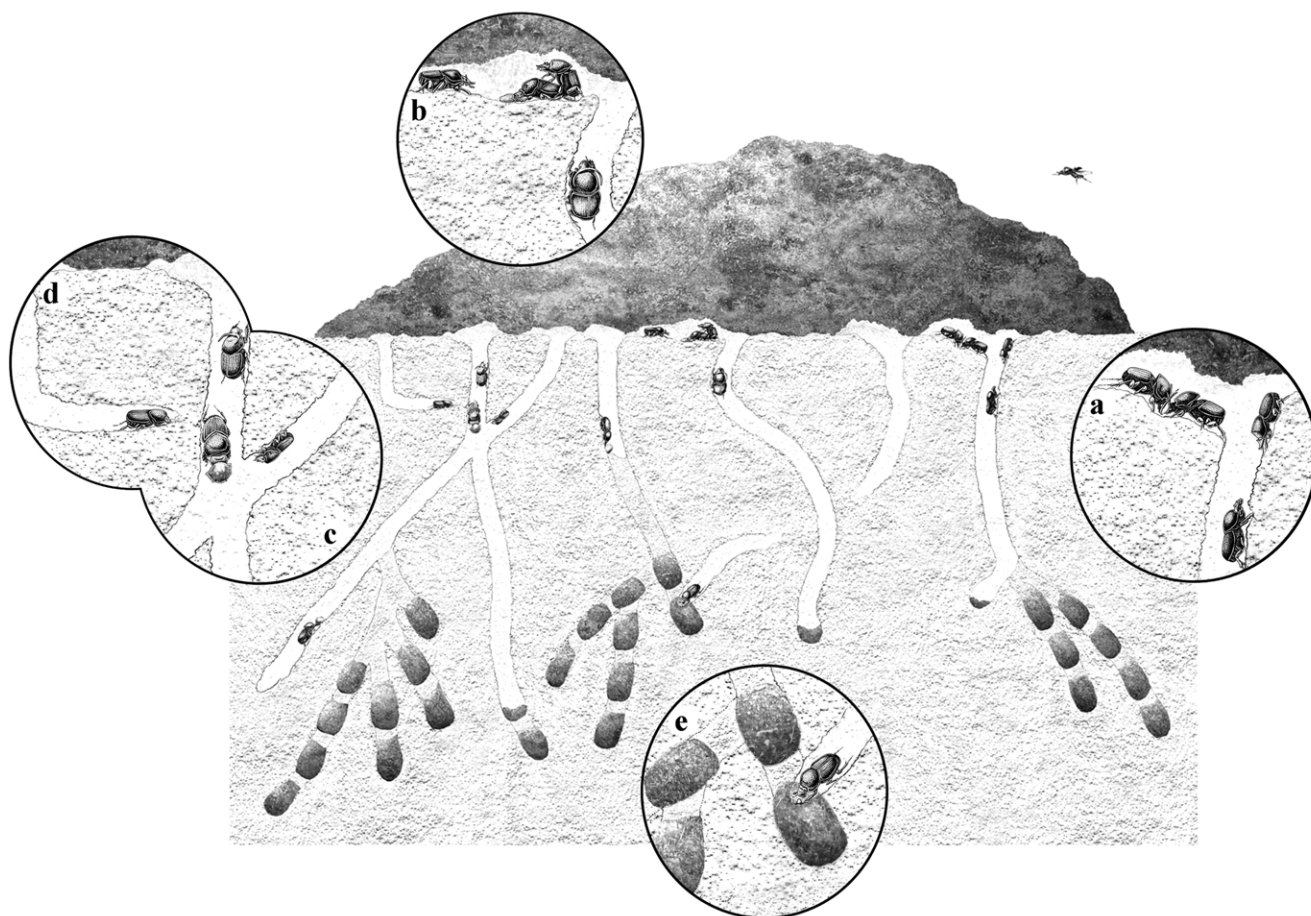
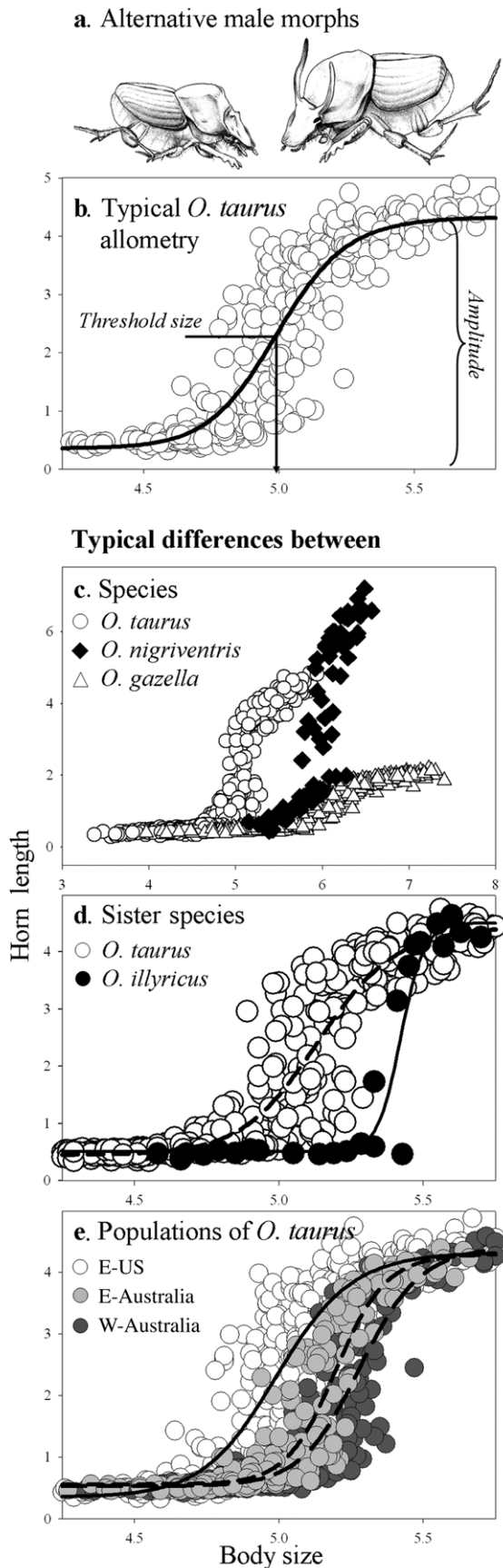


Figure 3. Mating system and alternative reproductive tactics in males and females of the horn polyphenic beetle *Onthophagus taurus*. Adult beetles colonize dung pads and dig tunnels into the soil underneath, creating a complex, interconnected tunnel system. Females pack dung into the blind ends of tunnels to provision food for their offspring in the form of brood balls. Each brood ball contains only one egg and constitutes the sole amount of food available for a developing larva. Males compete with each other for access to females during tunneling and brood ball production. Once females stop producing brood balls, the males desert and the females fill the remaining tunnel space with the previously excavated soil. (a–d) Alternative male reproductive tactics: Large, horned males defend tunnel entrances against rival males using their horns as weapons. Small, hornless males employ alternative sneaking behaviors to gain access to females when confronted with a physically superior opponent. Sneaking behaviors include (a) passing guarding males engaged in fights and (b) waiting near tunnel exits for females that collect dung for brood balls and mating aboveground with these females while the guarding males remain inside tunnels. Hornless males can also gain access to breeding tunnels and females underneath guarding males by (c) using tunnel interceptions created by the digging activity of breeding females and (d) actively digging horizontal side tunnels to intercept breeding tunnels. (e) Females also engage in alternative reproductive tactics: females typically reproduce by provisioning dung for larvae in the form of brood balls at the end of tunnels, but will parasitize brood balls produced by another female if given the opportunity. Figure reproduced from Ananthakrishnan TN, Whitman D, eds. Phenotypic Plasticity in Insects: Mechanisms and Consequences, Science Publishers, Inc., Enfield, NH (forthcoming); drawings by Barrett Klein.

Onthophagus taurus is native to the Mediterranean, but was introduced to several exotic locations, including an accidental introduction to the eastern United States as well as deliberate introductions to control cow dung and dung-breeding flies in eastern and western Australia (Moczek 2003). All three introductions occurred in the late 1960s, and on the basis of archival entomological collections, we now know that the ancestral Mediterranean populations that seeded these in-

troductions used the same average threshold body size to separate between hornless and horned male morphs.

Fast-forward to 1996. By this time I was beginning graduate studies at Duke University and was already familiar with US populations of *O. taurus*, including male allometries, which were basically the same wherever I looked in the United States. It therefore did not occur to me to explore other more distant populations until I read a manuscript by John Hunt



and Leigh Simmons (1997) from the University of Western Australia, which examined degree and patterns of asymmetry in beetle horns, using western Australian populations of *O. taurus*. A graph included in their manuscript depicted the average scaling relationship between body size and horn length of males, and even though the morphological landmarks the authors used were different from mine, the overall shape of their published allometry immediately suggested that there are some major differences between US and western Australian populations. Several experiments and thousands of beetle measurements later, we now know that both populations have indeed diverged substantially in their allometries—and most dramatically in their body size thresholds—relative to their Mediterranean ancestors: eastern US populations have evolved significantly smaller threshold body sizes, whereas their western Australian counterparts have evolved in the opposite direction, toward significantly higher threshold body sizes (figure 4; Moczek and Nijhout 2003). Differences between these populations persisted under common conditions in the laboratory for many generations, which confirmed that the observed allometric divergence indeed represented evolved, rather than environment-induced, differences between US and western Australian populations (Moczek 2003).

What was most exciting, however, was that divergences between eastern US and western Australian *O. taurus* populations mirrored, both in kind and magnitude, differences that are normally observed only between sister species or between species within the same genus (figure 4). This raised the possibility that exotic *O. taurus* populations have been undergoing evolutionary modifications similar to those that have helped past populations and species generate the allometric diversity present today. It also suggested that whatever caused these divergences, it could do so over extraordinarily short time periods.

What could have caused exotic populations of *O. taurus*, and perhaps other species before them, to evolve such divergent allometries? I hypothesized that the answer to this question might be found in the behavioral context within which male morphs functioned. If this context changed differently in different populations, this may help explain the evolution of novel and highly divergent allometries in these populations. In the meantime, I had found that a third population, estab-

Figure 4. Early allometric diversification between *Onthophagus taurus* populations mirrors divergence patterns in the genus as a whole. (a) Typical hornless and horned male morphs and (b) typical sigmoid scaling relationship between body size (x-axis) and horn length (y-axis). (c) Comparisons to other *Onthophagus* species suggest that evolutionary changes in amplitude and threshold size (defined in [b]) have played an important part in the morphological diversification of *Onthophagus* beetles. (d) Comparisons between sister species suggest that threshold divergence may mark the first step in allometric diversification. (e) Allometric divergence between three rapidly diverging populations of *O. taurus*.

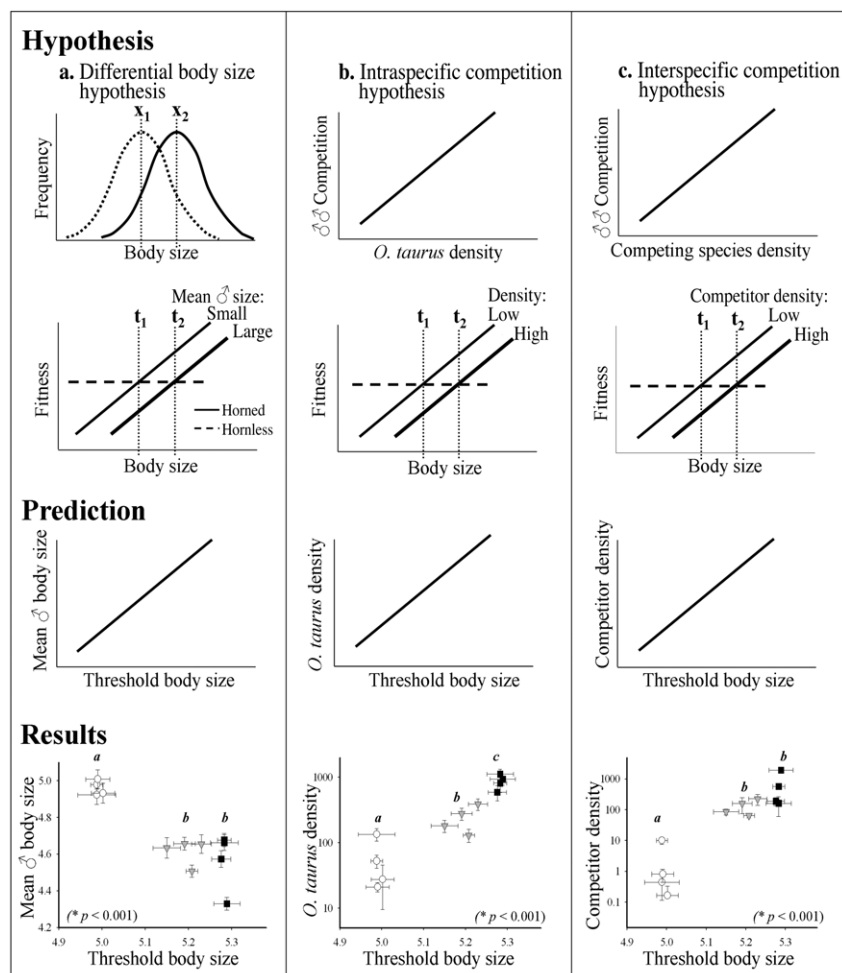


Figure 5. Three hypotheses concerning ecological mechanisms of threshold evolution in onthophagine beetles (the first two rows), predictions based on the hypotheses (third row), and actual results obtained from natural populations (fourth row). (a) According to the differential body size hypothesis, changes in the mean body size of competing males (x_1 to x_2) can change the competitive status of individual males relative to that of other males with which they compete for mates (row 1). In a population in which males are, on average, relatively small, males of an intermediate body size may be relatively large compared to their local competitors and able to maximize their fitness through fighting and the development of horns above a critical size, t_1 (row 2). In a population in which males are relatively large, these same intermediate-sized males may now be relatively small compared to their local competitors and may maximize their fitness by remaining hornless and engaging in sneaking behaviors up to a larger critical size, t_2 (row 2). This hypothesis predicts a positive correlation between male threshold body sizes and mean male body size in a population (row 3). Data from natural threshold-divergent populations fail to support this hypothesis (row 4). Even though these populations also exhibit highly significant differences in average male size (indicated by italic lowercase letters), the differences are in a direction opposite to what is predicted by the hypothesis. (b) According to the intraspecific competition hypothesis, increases in local densities of beetles increase male–male competition for females by increasing male encounter rates and decreasing the number of actively breeding females through interference competition (row 1). As local densities increase, male–male competition intensifies, and sneaking behavior becomes more profitable than fighting behavior over an increasing range of male body sizes. The increased profitability of sneaking behavior, in turn, selects for a shift of the critical threshold body size, t_p , to a larger body size, t_2 (row 2). This hypothesis predicts a positive correlation between male threshold body sizes and local *Onthophagus taurus* densities (row 3), which is strongly supported by data collected from three natural, threshold-divergent populations (row 4). (c) According to the interspecific competition hypothesis, increasing competition from other dung beetle species for dung should indirectly intensify male–male competition (row 1) by increasing the proportion of females that are unable to breed because of resource limitation imposed by the presence of competing species (row 2). This hypothesis predicts a positive correlation between threshold body sizes of male *O. taurus* and the densities of competing dung beetle species (row 3), which is supported in part by data collected from natural populations (row 4). Modified from Moczek (2003).

lished in eastern Australia at the same time as the other introductions, had also evolved higher threshold body sizes away from its Mediterranean ancestor and in the same direction as its western Australian counterpart, but to a much lesser degree. I decided at this point that three exotic populations that had diverged to different degrees from each other in their allometries provided enough of a toolbox to start looking for ecological factors that could explain the direction and magnitude of these divergences.

In particular, I decided to test three different, but not mutually exclusive hypotheses (figure 5). First, the *differential body size hypothesis* builds on the observation that a male's ability to compete with rival males is determined in part by his body size relative to that of his competitors (Emlen 1997). In a population in which the average male size is relatively small, genotypes that switch to the horned morph at a relatively small threshold body size should be favored. Individuals in populations in which the average male size is relatively large are instead predicted to delay the switch to a relatively larger body size. This should be manifest as a positive correlation between male threshold body sizes and mean male body size across different populations. Comparing average male body size across wide geographic ranges in the

eastern United States and in eastern and western Australia revealed major and highly significant differences between exotic ranges, but in a direction opposite to that predicted by the hypothesis (Moczek 2003). Western Australian males were on average smaller than their eastern US counterparts, and not larger as predicted by this hypothesis, which could therefore be rejected as an explanation (figure 5).

The second hypothesis argued that rather than differences in male size, differences in local densities of competing males (for females) and females (for breeding opportunities) could lead to divergence in threshold body sizes between populations. This *intraspecific competition hypothesis* proposes that under high density conditions only a fraction of females actually manage to breed, and males compete with each other for a relatively reduced pool of actively breeding females; and under such conditions only the largest males have the strength to benefit from fighting, and hence the possession of horns. Sneaking, in turn, should become profitable over a wider range of body sizes, and together these factors should favor genotypes that delay horn expression in all but the largest body sizes. This hypothesis therefore predicts a positive correlation between local threshold body sizes and local population densities, which was precisely what was observed in the three exotic ranges of *O. taurus*. Western Australian populations exhibited local densities two orders of magnitude higher than those of their eastern US counterparts, and eastern Australian populations fell in the middle, exactly as predicted by the hypothesis (figure 5; Moczek 2003).

Last is the *interspecific competition hypothesis*, according to which differences in the intensity of competition imposed by other species could also lead to divergences in threshold body sizes between populations. The idea behind this hypothesis is that the presence of other species reduces the amount of resources, such as the dung necessary for females to breed, thereby reducing the number of actively breeding females and thus intensifying male–male competition for the few females that do manage to breed under such adverse conditions. As before, increased levels of male–male competition should in turn limit the profitability of fighting behavior so that it benefits only the largest males, and favor a shift of the threshold body size to relatively larger body sizes. The interspecific competition hypothesis thus predicts that interspecific competition should be higher in populations with relatively high threshold body sizes. This hypothesis, too, was supported by highly significant differences in the predicted direction between Australian and eastern US populations (Moczek 2003).

Combined, these results suggested that differences in the intensity of intra- and interspecific competition could indeed drive allometric divergences between populations. Most significantly, however, these results suggest that relatively simple and probably ubiquitous ecological differences between populations may be all that it takes to initiate substantial and rapid morphological divergence in scaling relationships between such populations. If correct, this would suggest that novel thresholds may evolve much more easily, and probably much more frequently, than previously appreciated. For this

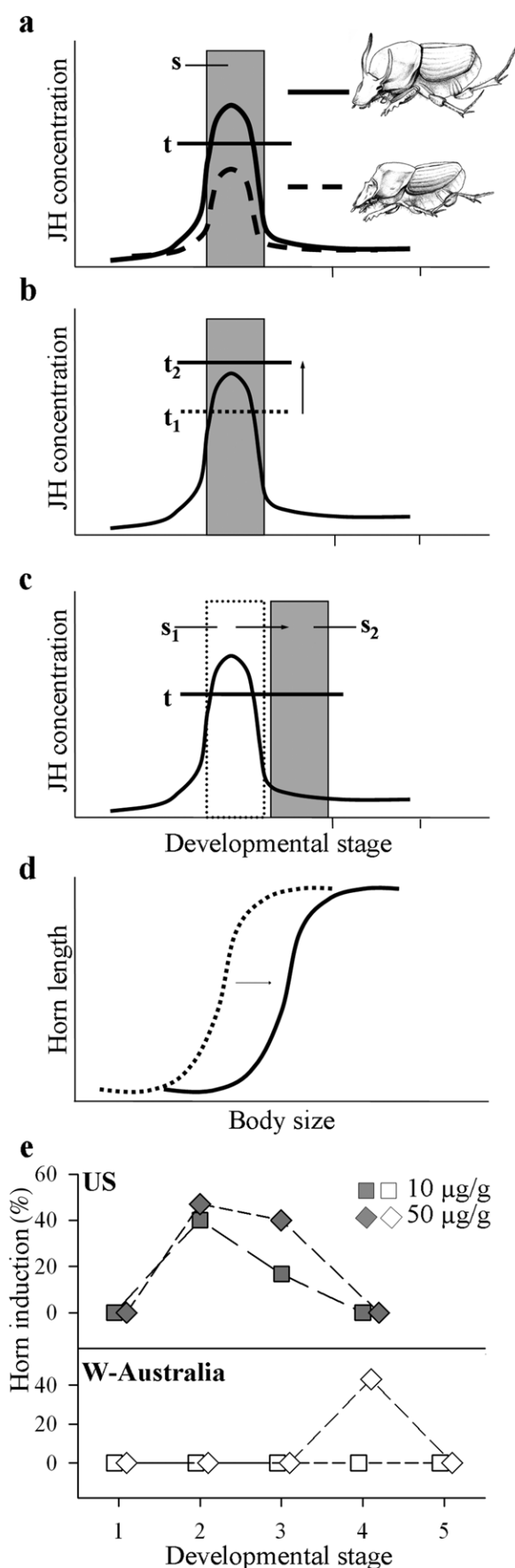
to be possible, however, the developmental machinery that controls horn growth would somehow have to be amenable to surprisingly rapid evolutionary modifications.

How to know when to grow?

What are the developmental mechanisms that regulate horn growth, and how have these mechanisms been modified to permit such rapid divergence between Australian and US populations of *O. taurus*? The first clues toward an answer emerged when both populations were reared side by side under the same conditions (Moczek and Nijhout 2002). For both the US and the Australian populations, only male larvae that exceeded a certain larval threshold weight expressed horns as adults. However, Australian populations turned out to have a significantly higher larval weight threshold than their US counterparts. Unexpectedly, Australian individuals also turned out to require over 30% more time to complete the third and last larval instar (Moczek and Nijhout 2002). These observations suggested that the rapid evolution of the threshold weight for horn expression in these populations may in some ways be linked to evolved changes in larval weight thresholds, but also to changes in timing of developmental events during the last instar.

But what are the developmental mechanisms that could link changes in larval weight thresholds and developmental timing to changes in threshold body sizes for horn expression? Earlier work by Emlen and Nijhout (1999) at Duke University suggested that the answer to this question may lie in the hormonal regulation of horn development. In particular, they suggested that juvenile hormone (JH), a universal and powerful endocrine regulator of insect development (Nijhout 1994), also mediates the size-dependent expression of horns in beetles. Emlen and Nijhout (1999) showed that *O. taurus* larvae destined to become hornless adults would develop horns if treated with the JH analog methoprene during a particular sensitive period during late larval development. Their results led them to postulate 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. They suggested that only male larvae heavy enough to express JH titers that exceeded 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 (figure 6). While their model is simple and most likely incomplete, it suffices to generate hypothetical scenarios as to how the critical body size that separates horned and hornless males could change (Moczek and Nijhout 2002; see Emlen and Nijhout 2001 for a possible expansion of this endocrine model).

For example, if the JH threshold required for horn induction were elevated, medium-sized males would no longer produce a JH titer necessary for horn expression, and hence would develop into the hornless morph (figure 6b). Alternatively, a change in the timing of the sensitive period relative to JH secretion would also modify the critical threshold



body size. For example, if the sensitive period were delayed to just after the hormone peak, medium-sized males again would express titers below the threshold, and would therefore develop into hornless males (figure 6c). On the level of a population, both developmental modifications would be capable of shifting the body size threshold to larger body sizes (figure 6d). I decided to approach these hypotheses experimentally by inducing horns in incipient hornless male larvae of western Australian and eastern US strains, varying both hormone concentration and timing of application. The results were surprisingly clear-cut: horn induction in western Australian males was delayed subtly but significantly—by 24 to 48 hours—and western Australian males required slightly, but again significantly, higher hormone dosages for horn induction than their eastern US counterparts (figure 6e; Moczek and Nijhout 2002). These results supported the hypothesis that relatively minor changes in the sensitivity to JH and its timing during larval development may be the only developmental modifications required to allow beetle populations with different ecological conditions to evolve novel allometries.

These results are also significant because they point toward at least some answers regarding one of the major questions posed at the beginning of this article: How do ecological, genetic, and developmental mechanisms interact during the early stages of morphological diversification? However, these results

Figure 6. Endocrine mechanisms of threshold evolution in onthophagine beetles. (a) Endocrine control of male horn dimorphism (modified from Emlen and Nijhout 1999). Males are thought to differ in juvenile hormone (JH) titers as a function of their body size. Only males that exceed a certain size or weight express JH titers above a threshold (t) during a particular sensitive period (s), and in response will develop horns as adults. Smaller males with JH titers below this threshold will remain hornless. (b) Elevation in the JH threshold (t_1 to t_2) prevents medium-sized male larvae from expressing JH titers above the threshold necessary for horn development. Such larvae will now develop into the hornless instead of the horned morph as adults. (c) A delay in the sensitive period for JH (s_1 to s_2) causes the JH titer of medium-sized male larvae to fall below the JH threshold necessary for horn induction before horn primordia become JH sensitive. As in (b) above, such males will now develop into the hornless instead of the horned morph as adults. (d) Both developmental modifications can generate a population-wide shift of the critical threshold body size to larger body sizes. (e) Experimental results from two threshold divergent populations (eastern United States and western Australia) support both hypotheses. Male larvae that normally would develop into the hornless morph were subjected to applications of the JH analog methoprene at different time points during late larval development (x-axis). The y-axis indicates the percentage of males that responded to the treatment by switching developmental fate and developing horns. Horn induction in Australian males requires higher dosages (50 instead of 10 micrograms per gram) and exhibits delayed sensitivity compared with horn induction in US males. Modified from Moczek and Nijhout (2002).

do not help us with the first question, namely how phenotypic novelties, such as beetle horns, originate in the first place. To address this question, we need to know more about where horns come from developmentally and genetically. For example, what are the developmental and genetic steps in the building of a horn during larval ontogeny? And are these steps themselves novel innovations, or have they been borrowed from preexisting developmental processes and then re-assembled to give rise to a novelty? As we shall see below, even though studies are just beginning to answer these questions, they are already providing surprising insights into the economy and ingenuity of innovation and diversification in horned beetles.

A horn is a leg?

The growth of horns is a remarkably dynamic process, confined largely to an approximately 48-hour time window very late in larval development, when the animal is about to molt into a pupa (Moczek and Nagy 2005). Externally, individuals resemble regular, albeit passive, larvae at this stage, yet internally these individuals are undergoing dramatic and widespread remodeling, and scientists therefore refer to this as a separate, prepupal stage.

The onset of the prepupal stage is characterized by two major events: (1) massive, widespread cell death of muscle tissue inside the animal's head and thorax and (2) a beginning detachment of the larval epidermis from the cuticle (figure 7; Moczek and Nagy 2005). The cuticle is the outermost non-living layer, composed mainly of chitin, that makes up the hard exoskeleton of insects, whereas the epidermis is the outermost layer of living cells directly below the cuticle (Snodgrass 1935). As soon as epidermal detachment has been achieved, the cells of certain epidermal regions begin to divide at a high rate, causing the epidermis to grow very quickly in certain locations. Because this epidermal growth occurs right underneath the larval cuticle, it is not visible from the outside. Furthermore, the resulting new tissue cannot yet expand and instead undergoes massive upfolding underneath the larval cuticle. Once the animal is ready to molt into a pupa and sheds its larval skin, the folded-up horn tissue becomes free to telescope outward and to form the pupal and future adult horn (figure 7; Emlen and Nijhout 1999, Moczek and Nagy 2005).

As remarkable as this process seems, it bears a striking resemblance to the way many insects grow their more traditional appendages, such as legs, mouthparts, or antennae (Fristrom and Fristrom 1993). In most insects, all of these appendages originate from very similar epidermal outbuddings, with the only deviations being found in the appendages of the so-called higher flies (like the fruit fly *Drosophila*) and the wings of butterflies, which originate from cell clusters called imaginal disks that are specified and set aside during embryonic rather than late larval development (Nijhout 1991). Apart from these exceptions, all other insects grow their legs, mouthparts, and antennae the way horned beetles grow their horns (Nagy and Williams 2001). Could it be that horn development and differentiation may be regulated by some of the same

genetic mechanisms that also coordinate the development of more traditional appendages?

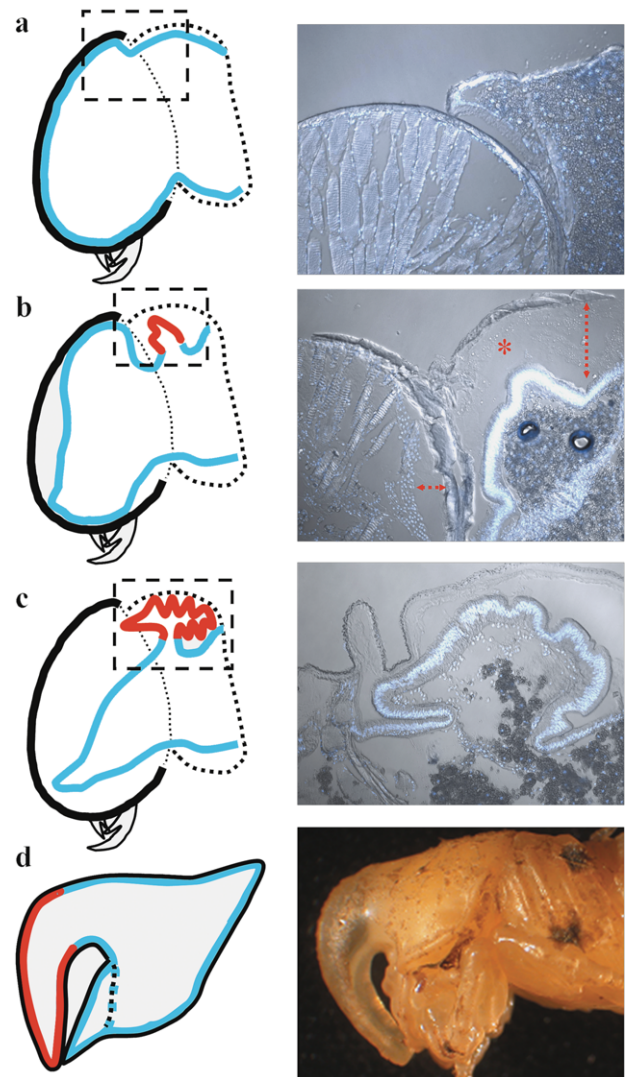


Figure 7. Beetle horns grow explosively during an approximately 48-hour time window at the end of larval development. **Left:** schematic highlighting prepupal thoracic horn growth. **Right:** corresponding sagittal sections of head capsule and first thoracic segment of a presumptive horned male *Onthophagus nigriventris* stained with DAPI to highlight cell nuclei (blue dots). (a) Before the prepupal stage, the larval epidermis (blue) fully lines the larval cuticle (black). (b and c) At the onset of the prepupal stage, the larval epidermis detaches from the cuticle (indicated by arrows), and selected regions (indicated by an asterisk) undergo rapid cell proliferation. The resulting tissue folds up underneath the larval cuticle. During the second half of the prepupal stage, the epidermis secretes the future pupal cuticle. (d) Once the animal molts to a pupa, the folded epidermis and future pupal cuticle are free to expand, harden, and form the pupal thoracic horn. Modified from Moczek and Nagy (2005).

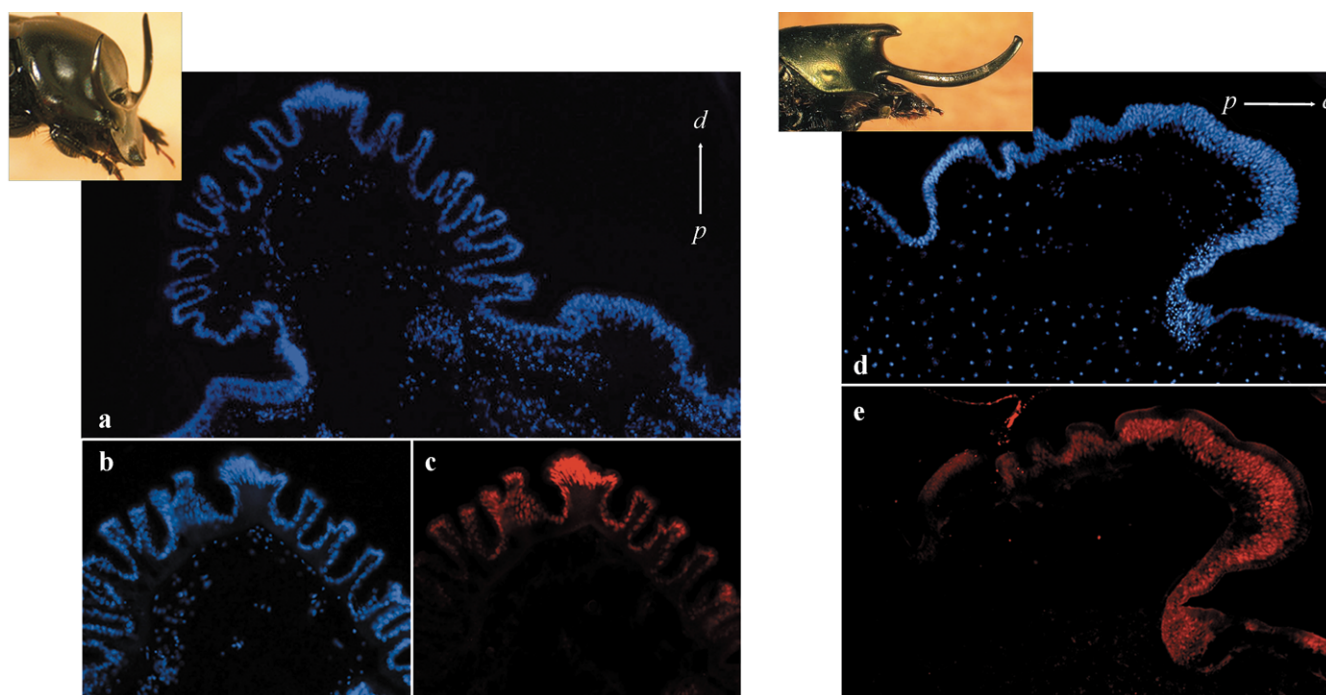


Figure 8. Examples of genes expressed during the development of beetle horns. **Left:** Transverse section through one of two head horn primordia in *Onthophagus taurus*: (a) overview and (b) detail, stained with DAPI to highlight the nuclei of cells that make up the epidermal cell layer. Note the intense, mushroom-like folding of the horn primordium. Orientation of the proximodistal axis is indicated by arrow between p (proximal) and d (distal). (c) The same section as in (b), stained with anti-Dll antibody. Bright red nuclei indicate cells in which the Dll gene is expressed. Expression is most intense in those cells that will give rise to the distalmost part of the future horn, consistent with a conservation of function of the Dll gene compared to its role in the formation of traditional appendages. **Right:** Sagittal section through the thoracic horn primordium in *Onthophagus nigriventris*. (d) DAPI staining to highlight nuclei of cells that make up the epidermal cell layer. (e) The same section as in (d), stained with anti-aristaless (al) antibody. Bright red nuclei indicate cells in which the al gene is expressed. At this stage of development, al expression can be observed in the greater distal area of the developing horn primordium. Modified from Moczek and Nagy (2005).

The coordination and regulation, or patterning, of appendages has been studied intensely in a variety of insects, and also in noninsect arthropods such as spiders and crustaceans (Panganiban et al. 1997, Nagy and Williams 2001, Kojima 2004). One of the major conclusions that can now be drawn from these studies is that a remarkable degree of diversity in appendage types has evolved despite an apparent conservation of the regulatory mechanisms that help specify appendage architecture. For example, all appendages are characterized by a proximal (close to the body) and a distal (away from the body) region, and a corresponding proximodistal axis (Panganiban et al. 1997). The expression of and interactions between three genes, *Distal-less* (*Dll*), *homothorax* (*hth*), and *extradenticle* (*exd*), appear to represent a nearly universal regulatory mechanism for specifying this axis, which has now been documented in a wide range of insects and noninsect arthropods (Kojima 2004). Simply speaking, *Dll* expression induces a distal fate; that is, *Dll*-expressing cells will give rise to distal appendage regions such as the tibia and tarsal segments of the leg or the maxillary palps, and experimental interference with *Dll* expression prevents the development of these appendage regions (Kojima 2004). Ex-

pression of the *hth* gene, on the other hand, results in a protein that binds to the *exd* protein and allows it to enter the nucleus, where *hth* protein and *exd* protein act cooperatively in the subsequent regulation of other genes. As a consequence, even though *exd* is initially expressed throughout the entire epidermis, *exd* protein localizes only into nuclei of those epidermal cells that also express *hth*. Such cells are thereby fated to give rise to proximal appendage regions, including those where the appendage will be anchored into the body wall (Kojima 2004).

Given the congruence in patterning mechanisms across a wide range of appendage types, I hypothesized that proximodistal axis formation during beetle horn development may also be patterned by *Dll*, *hth*, and *exd*, a hypothesis that turned out to be correct (figure 8).

Regardless of location, number, or final shape of a horn, the epidermal cells that express *Dll* during detachment and unfolding during the prepupal stage will give rise to the distalmost region of the future adult horn (figure 8; Moczek and Nagy 2005), while nuclear *exd* and *hth* expression is observed only in incipient proximal horn regions. With respect to the specification of its proximodistal axis, a beetle horn is

thus nothing novel; instead, it appears to be the product of the redeployment, or co-option, of an ancient, preexisting de-

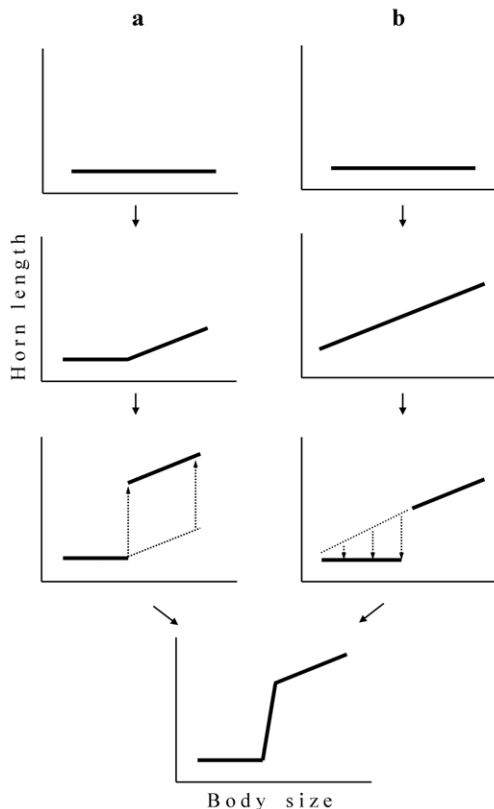


Figure 9. Alternative hypotheses for the origin of facultative, size-dependent horn expression in male beetles. (a) According to the first hypothesis, male horn dimorphism may have originated from uniformly hornless ancestors. Genotypes initially expressed no horns regardless of body size, but subsequently evolved the ability to express horns in males that exceeded a certain body size. The ability to induce horn growth above a certain size threshold increased over time. This hypothesis predicts that horn-inductive developmental events occur only in large males. (b) The second hypothesis also assumes that horn dimorphisms originated from uniformly hornless ancestors. Genotypes then evolved the ability to express horns in all males proportional to their body size. At this stage, small males were simply scaled-down versions of large males. Genotypes subsequently evolved developmental mechanisms permitting them to repress horn development in males below a certain body size. The ability to repress horns below and induce horns above a certain size threshold then increased over time. This hypothesis suggests that at least some horn-inductive events may be taking place in all males regardless of body size, but that horn induction may be incomplete or secondarily repressed in small males only. The expression of at least some horn regulatory genes, such as *Distal-less*, in the rudiments of small males supports the second hypothesis (Moczek and Nagy 2005).

velopmental mechanism. However, *Dll*, *hth*, and *exd* are merely three of a large number of patterning genes involved in appendage formation. To date, my research group has been able to investigate close to a dozen appendage genes in the context of beetle horn development, including some whose expression patterns actually suggest a loss, or modification, of function during horn development compared to their traditional role during development of more typical appendages. Although it is still too early to quantify the respective contributions of conserved and modified appendage patterning mechanisms in the evolution of beetle horns, these results suggest that the origin of horns probably involved the selective, rather than wholesale, co-option of traditional appendage patterning mechanisms, some of which continued to maintain a conserved function in the development and evolution of a novel appendage, while others did not.

A horn is a horn is a horn?

Among arthropods, including beetles, different body regions are unambiguously defined by particular appendages, such as different mouthparts for the various head segments, legs for each of the three thoracic segments, and wing covers and wings for the second and third thoracic segments (Snodgrass 1935). Horns, however, can grow anywhere—from the very tip, sides, or back of either the head or the thorax, or both (Moczek 2005). Are horns that grow in different body regions products of the same sequence of genetic and developmental processes taking place in different locations, or are horns growing on the head somehow different from horns that grow from the thorax? Analysis of the developmental regulation of beetle horns has recently begun to provide some of the first evidence that favors the second perspective, and with it a major and surprising implication: different horns may have different developmental origins, and most likely different evolutionary histories.

One type of evidence comes from gene expression studies. An important patterning gene during appendage development is the transcription factor *aristaless* (*al*), which represents a major target of the epidermal growth factor receptor (EGFR) signaling pathway. EGFR signaling represents an alternative means of establishing a proximodistal axis through the formation of steep gradients of some of its components, which in turn differentially activate target genes such as *al* (Campbell 2002, Galindo et al. 2002). In the legs and antennae of flies, *al* expression is crucial for the patterning of the extreme tip of the future appendage, and a similar role has been suggested for the legs, mouthparts, and cerci of crickets (Campbell 2002, Miyawaki et al. 2002, Kojima 2004). Beetles, too, share similar distal *al* expression patterns in their mouthparts and antennae, yet only in one type of horn: thoracic horns are characterized by extensive and strong *al* expression in the distal region of the developing horns, whereas head horns show no signs of *al* expression (figure 8; Moczek and Nagy 2005). While functional analyses have yet to confirm a functional role of *al* expression in thoracic horn development, these data do suggest strongly that different types of horns may be patterned

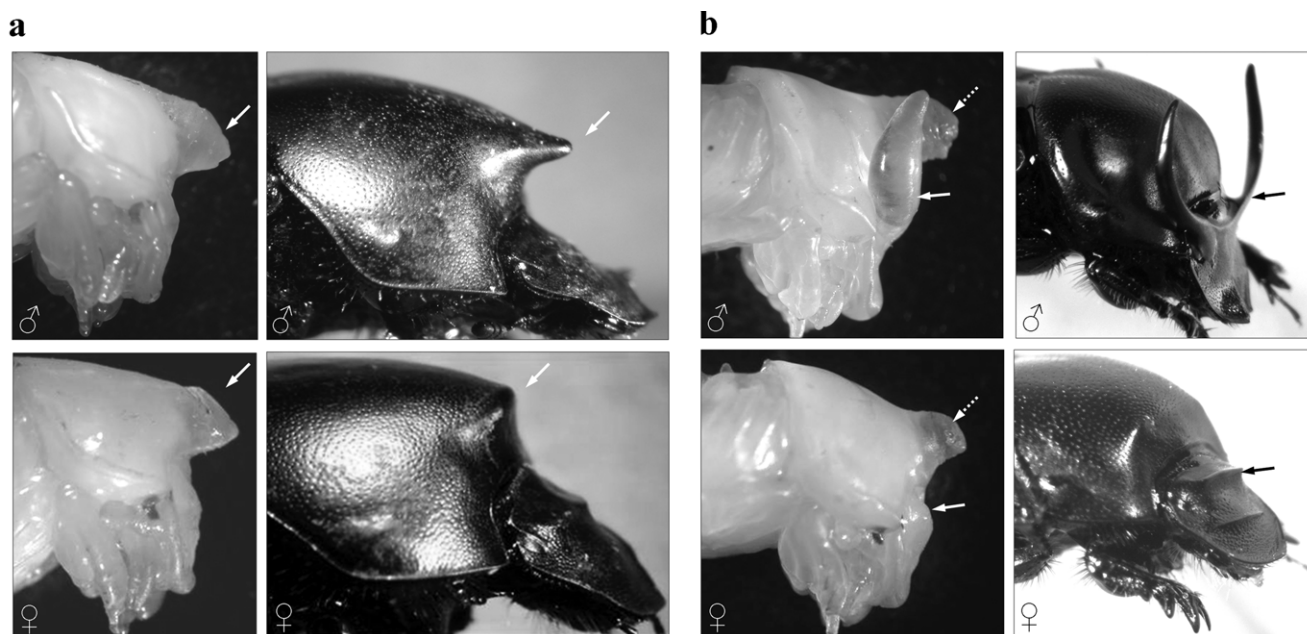


Figure 10. Sexual horn dimorphism has multiple developmental and evolutionary origins. (a) Female horned beetles could have expressed horns ancestrally, but then lost them secondarily. If this hypothesis is correct, developmental remnants of female horn expression might still be detectable. Female *Onthophagus nigriventris* pupae express a temporary thoracic horn (bottom left) similar in size and shape to that of their male counterparts (top left, highlighted by solid arrow) but reabsorb most of it before they molt to become adults (shown to the right of corresponding pupa). Females also share similar patterns of regulatory gene expression (not shown; Moczek and Nagy 2005). In combination, this suggests that female hornlessness in this species may have involved the secondary sex-specific loss of horns from an originally horn-monomorphic ancestor. (b) Alternatively, the absence of horns in females could be a reflection of the ancestral character state; that is, horns evolved right away as a male-specific trait. Sexual dimorphism in head horns in species such as *Onthophagus taurus* may have originated in this fashion. Female *O. taurus* (bottom right) show no signs of horn-inductive developmental events (such as pupal head horn growth or expression of patterning genes) in the same tissue regions that give rise to paired head horns in their male counterparts (highlighted by solid arrow). Note that male and female *O. taurus* also grow a large thoracic horn (indicated with striped arrows) clearly visible in pupae. Like female *O. nigriventris* described above, female and male *O. taurus*, however, reabsorb their entire thoracic horn during the pupal stage and molt into adults that lack any indication of the past existence of a thoracic horn. Modified from Moczek and Nagy (2005).

by different sets of patterning mechanisms, a conclusion that has recently received further support from investigation of other EGFR signaling components. A second type of evidence in favor of this hypothesis emerges when we explore the developmental regulation of intraspecific diversity in horn expression—that is, how females and small males manage not to grow horns in most species.

Different ways not to grow a horn

Two hypotheses have been proposed regarding the origin of male horn dimorphism (figure 9; Moczek 2005). Male horn dimorphism could have originated from an ancestor in which all males were hornless. According to this hypothesis, male horn dimorphism appeared when genotypes emerged with the ability to induce horn expression in males that exceeded a certain size threshold. If this hypothesis is correct, then evidence of horn induction, such as tissue proliferation and the expression of patterning genes, should be confined to large males only.

Alternatively, male horn dimorphism could have originated from an ancestor in which all males grew horns regardless of size, and in which large males were simply enlarged versions of small males. Then genotypes somehow acquired the ability to inhibit full horn growth in males smaller than a critical size threshold (figure 9). If this hypothesis is correct, then there should be evidence of horn induction in all males regardless of size, and possibly additional evidence of either incomplete activation or repression of horn growth in small males only.

The same logic can of course be applied to sexual dimorphisms, that is, to the absence of horns in females. Sexual horn dimorphism could have arisen from an ancestor in which both sexes were hornless (and then horns arose as a male-specific trait) or from an ancestor in which both sexes were initially horned (and female hornlessness evolved via a secondary loss through inhibition of horn growth in females). Again, only in the latter case would we expect to see signs of horn growth or patterning in females.

For male horn dimorphism, the answer is clear-cut and the same across species and horn types: small males show signs of horn growth and patterning, just like their larger counterparts, suggesting that facultative, size-dependent male horn dimorphisms are indeed a secondary modification of obligate horn growth regardless of male body size (Moczek and Nagy 2005). For sexual horn dimorphisms, however, the case is less clear, and different hypotheses are supported for different horn types (figure 10). Male head horns, as in *O. taurus*, appear to develop in a strictly sex-specific manner. Females never show any signs of epidermal proliferation or expression of horn patterning genes during prepupal development in this species, suggesting that female hornlessness (for head horns) is most likely ancestral (Moczek and Nagy 2005). Not so for thoracic horns, as in *Onthophagus nigriventris*. In this species, females not only actively express the same genes in the same body regions as their male counterparts, they actually grow a thoracic horn during the prepupal stage (Moczek and Nagy 2005). This horn is compatible in shape and, with the exception of the very largest males, also in size to that of their male counterparts, but unlike males, who retain their horn into adulthood, females reabsorb their entire horn during the pupal stage and before the final molt to the adult stage (figure 10). This result was most surprising and unexpected, yet consistent with the hypothesis that ancestrally both sexes may have expressed thoracic horns in this species, and that females lost their horns by evolving mechanisms to un-grow them, rather than not growing them in the first place. In combination, these data also suggest that thoracic and head horns may differ not only in the regulation of their initial growth but also in subsequent mechanisms used to generate sexually dimorphic adults (Moczek 2005). The notion that different types of horns may have evolved independently in the same clade has recently received further support from the first partial phylogenetic analysis of the genus *Onthophagus* (Emlen et al. 2005). This and future phylogenetic analyses of *Onthophagus*, and of other taxa that contain horned beetles, are likely to provide a crucial framework for future comparative developmental and ecological approaches into the origins of diversity in these organisms.

Now you see me, now you don't

A particularly striking case of horn loss may have occurred in the now familiar *O. taurus* (figure 10b). As it turns out, the head horns that develop in large male prepupae are not the only type of horns produced in this species: all males and females, regardless of body size, also grow a large thoracic horn (Moczek and Nagy 2005). In fact, their thoracic horn is larger than that of many species that retain this horn into the adult stage. *Onthophagus taurus*, however, loses its entire thoracic horn in both sexes and ends up with a hornless thorax in the adult (figure 10). This observation raises the possibility that *O. taurus*'s ancestor possessed a thoracic horn, possibly instead of or in addition to paired head horns, that was then lost for whatever reason. If correct, this scenario suggests that, as discussed above, the mechanism for losing the thoracic horn

was not to inhibit the process of making it in the first place, but rather to add a developmental step that reverses the growth that took place a few days earlier. Integrating phylogenetic and comparative developmental approaches should allow researchers to shed more light on these and related questions in the future.

The origins of diversity

I began this article by posing one of the most fundamental unresolved questions in evolutionary biology: How do ecological, developmental, and genetic mechanisms interact in the origin and diversification of novel traits? Over the past decade, studies on horned beetles and beetle horns have begun to address this question through a wide range of approaches. The genesis of diversity in horned beetles appears to have involved, on one side, the co-option and reorganization of pre-existing developmental mechanisms to build new phenotypes, such as the use of appendage patterning genes during horn development. At the same time, however, it also involved a great deal of innovation through the recruitment of modifier mechanisms to help shape intra- and interspecific diversity, such as the secondary loss of horns through the differential reabsorption of transient horn primordia. Finally, the biology of horned beetles highlights the importance of recognizing variability in external conditions, such as nutrition or social environments, as a creative force that defines and shapes organisms along with the environment in which they grow, develop, behave, and reproduce. Though clearly only beginning, studies on horned beetles and beetle horns already illustrate the feasibility of an integrative, multidisciplinary approach toward understanding the origins of organismal diversity.

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