

Hedgehog signaling enables nutrition-responsive inhibition of an alternative morph in a polyphenic beetle

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The recruitment of modular developmental genetic components into new developmental contexts has been proposed as a central mechanism enabling the origin of novel traits and trait functions without necessitating the origin of novel pathways. Here, we investigate the function of the hedgehog (Hh) signaling pathway, a highly conserved pathway best understood for its role in patterning anterior/posterior (A/P) polarity of diverse traits, in the developmental evolution of beetle horns, an evolutionary novelty, and horn polyphenisms, a highly derived form of environment-responsive trait induction. We show that interactions among pathway members are conserved during development of *Onthophagus* horned beetles and have retained the ability to regulate A/P polarity in traditional appendages, such as legs. At the same time, the Hh signaling pathway has acquired a novel and highly unusual role in the nutrition-dependent regulation of horn polyphenisms by actively suppressing horn formation in low-nutrition males. Down-regulation of Hh signaling lifts this inhibition and returns a highly derived sigmoid horn body size allometry to its presumed ancestral, linear state. Our results suggest that recruitment of the Hh signaling pathway may have been a key step in the evolution of trait thresholds, such as those involved in horn polyphenisms and the corresponding origin of alternative phenotypes and complex allometries.

co-option | modularity | developmental plasticity | allometry | threshold trait

Understanding the genetic and developmental mechanisms underlying the origin and diversification of novel, complex traits is a fundamental objective of evolutionary biology. The recruitment of modular developmental genetic components into new developmental contexts has been proposed as a central mechanism enabling the origin of novel traits without necessitating the origin of novel genes or developmental pathways (1, 2). At the same time, redeployment of “old” genes and pathways into novel developmental contexts has the potential to create critical new opportunities for morphological diversification: for example, the recruitment of appendage patterning genes has played a key role in the origin of butterfly eye spots, a remarkable evolutionary novelty (3). Moreover, by being placed in the context of wing patterning, the further evolution of eye spot formation could exploit preexisting patterning mechanisms, ultimately enabling eye spot formation to diversify depending on which wing, wing surface, or position it has occurred (4). Therefore, newly deployed gene regulatory networks can contribute to both macroevolution (e.g., butterfly eyespot generation) and microevolution (e.g., diversification of the eyespot pattern).

One particularly significant axis of diversification receiving growing attention from evolutionary and developmental biologists concerns environment-responsive trait formation (5, 6). Environment-responsive development or developmental plasticity is ubiquitous across trait types and taxa and creates especially significant evolutionary degrees of freedom for novel, complex traits (5, 7). Here, polyphenic development—the most extreme case of developmental plasticity, whereby environmental factors cue the

development of two or more discrete morphs or castes—has become increasingly recognized for its importance as a potential facilitator of adaptive radiations (8). For instance, in many butterfly species, seasonal conditions critically alter selective environments, and seasonal sensitivity in eye spot formation is able to adjust wing phenotypes, thereby maintaining high fitness across fluctuating environments (9). Similarly, environment-dependent induction of carnivory in spadefoot toad tadpoles (10, 11) or tooth formation and bacterial predation in nematodes (12), two striking and complex evolutionary novelties, greatly affect the adaptive significance of each innovation, thereby facilitating their adaptive radiations (13). Here, we investigate the functional significance of the Hedgehog (Hh) signaling pathway, a deeply conserved cellular transduction pathway, in the development of beetles horns, a striking evolutionary novelty (14), and specifically, the origins of environment-responsive horn formation, alternative male phenotypes, and body size thresholds, emergent phenotypes that have greatly impacted patterns of morphological radiation among horned beetles (15–17).

Several thousands of species of horned beetles exist worldwide, with the greatest diversity found in the rhinoceros (Dynastinae) and scarab (Scarabaeinae) beetles, two subfamilies that independently evolved horns. Beetle horns are primarily used as weapons in male competition (18), restricted to or greatly exaggerated in males (19), and in most species studied thus far, greatly affected in their development by the nutritional conditions experienced during the larval stage (20). Here, the evolution of nutritional responsiveness in the context of horn formation is thought to have played an

Significance

Complex, nongradual responses to environmental conditions are commonplace in nature and perhaps most extreme in polyphenic insects where continuous variation in nutrition experienced in early development gives rise to discrete alternative castes or morphs. This research shows that the hedgehog (Hh) pathway has acquired a novel and highly unusual role in the nutrition-dependent regulation of polyphenic development of a beetle. Experimental repression of Hh signaling returns a highly discontinuous response to nutrition to its presumed ancestral, gradual state. Our results suggest that recruitment of the Hh signaling pathway may have been a key step in the evolution of trait thresholds and the corresponding origin of alternative phenotypes and complex allometries.

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especially critical role in horned beetle diversification by (i) enabling the evolution of some of the most extreme examples of condition-dependent exaggerated male secondary sexual traits (21); (ii) facilitating the origin of horn polyphenisms and discrete hornless sneaker and horned fighter morphs adapted for alternative reproductive niches (18); (iii) establishing sharp threshold body sizes that decouple alternative developmental trajectories and enable the integration of two separate, complex, disparate male phenotypes from the same genome (16, 22); and (iv) contributing to the enormous radiation of horned beetles through the evolution of morph-specific morphologies, behaviors, and physiologies and the diversification of size thresholds, perhaps best illustrated in the >2,000 species-rich genus *Onthophagus* (16, 17, 19, 23).

Previous studies have shown that horn development is instructed by many of the same genes that regulate the formation of regular appendages, such as leg gap genes (14), that nutrition responsiveness of horn development may be coupled to insulin signaling (24, 25), and that sex- and morph-specific horn exaggeration may be facilitated by *doublesex* (*dsx*), a master regulator of somatic sex determination (23, 26, 27). However, how beetle horns achieve their strikingly dimorphic expression in many species and the developmental mechanisms that enable the definition of a sharp body size threshold separating alternate morphs from what is presumed to be a continuous range of nutritional conditions are entirely unknown. Here, we investigate the function of the Hh signaling pathway, a highly conserved pathway best understood for its role in patterning anterior/posterior (A/P) polarity in body segments and appendages (28, 29), in the development of horns, horn polyphenisms, and threshold body sizes. Specifically, we show that, although interactions among pathway members remain conserved and involved in regulating A/P polarity in traditional appendages, such as legs, the Hh signaling pathway has acquired a novel and highly unusual role in the nutrition-dependent regulation of horn polyphenisms by actively suppressing horn formation in low-nutrition males. Our results suggest that recruitment of the Hh signaling pathway may have been a key step in the evolution of trait thresholds, such as those involved in horn polyphenisms and the corresponding origin of alternative phenotypes and complex allometries.

Results and Discussion

To investigate the role of the Hh pathway in *Onthophagus* development, we first cloned fragments of *Onthophagus taurus* *hh* (the morphogen in the absence of which Hh signaling is deactivated), *patched* (*ptc*; the Hh receptor that inhibits Hh signaling unless bound to Hh protein), and *smoothed* (*smo*; a membrane protein that, in the absence of Hh protein, is constitutively inhibited by *ptc* but disinhibited in the presence of Hh, thereby activating the pathway) using sequence data from an earlier study (30). Below, we describe the effects of dsRNA injections beginning with their role in the regulation of general postembryonic development and followed by their function in the context of horn morphogenesis, focusing on nutrition-sensitive horn formation. We discuss the implications of our results for our understanding of the origin of environment-sensitive trait formation and the evolution of trait thresholds and nonlinear allometries.

Functional Significance of the Hh Pathway in Late *Onthophagus* Development. RNAi-mediated knockdown of *hh* and *smo* resulted in overall similar phenotypes, consistent with their presumed position within the Hh pathway and suggesting that the functions of both genes are at least partly overlapping (Fig. 1). After *smo*^{RNAi}, animals exhibited a survival rate of 32% (98 of 305). We observed developmental defects in legs and wings (56 of 64 males and 28 of 34 females showed defects in either one or both appendage types) (Fig. 1 and Fig. S1). For instance, compared with control-injected animals, the tibia and femur of *smo*^{RNAi} animals were more slender,

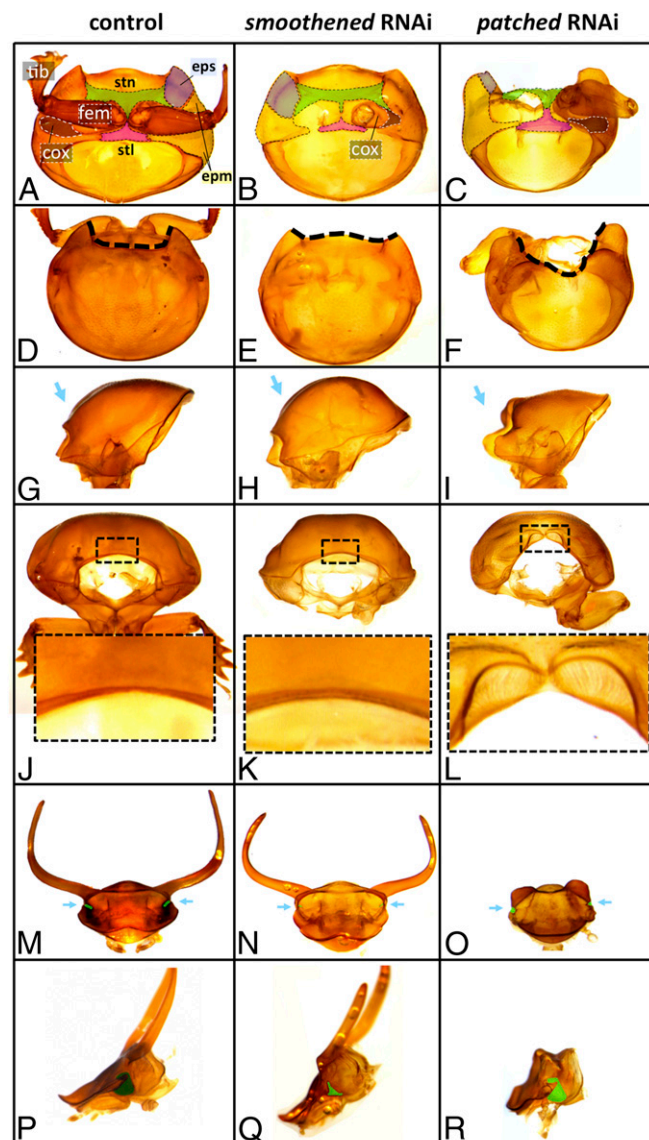


Fig. 1. Effect of *hh*, *smo*, and *ptc* dsRNA injection on *Onthophagus* development. Prothorax and head of (Left) control-injected, (Center) *smo*^{RNAi}, and (Right) *ptc*^{RNAi} animals are shown. (A–C) Comparison of the prothorax from the ventral side. The differences of *smo* and *ptc* function in prothoracic development are most obvious in the sternum (stn; green), episternum (eps; blue), and epimeron (epm; yellow). Note that in situ size of the coxa (area shaded black) is reduced in *smo*^{RNAi} individuals relative to control-injected and *ptc*^{RNAi} animals. (D–F) Development of the notum is also affected by RNAi. (E) Note that the anterior edge of the pronotum (thick dashed lines in D–F) extends anteriorly in *smo*^{RNAi} animals, whereas (F) *ptc*^{RNAi} results in significant reduction of the anterior pronotal edge. (G–I) Lateral view of the same prothoraces; *smo*^{RNAi} animals exhibit prominent bulging of the pronotum, whereas *ptc*^{RNAi} animals lack the anterior region (blue arrows). (J–L) Frontal view of the same prothoraces. (K) Effect of *smo*^{RNAi} was minimal on the anterior edge, whereas (L) *ptc*^{RNAi} resulted in development of ectopic bristles. (M–O) Frontal view of the head. Compound eyes are labeled in green and highlighted by blue arrows. Head shape was only lightly affected by RNAi. (N) All but one *smo*^{RNAi} animal developed full-sized horns, whereas (O) horns in all *ptc*^{RNAi} animals were vestigial. (P–R) Lateral view of the head. (Q) Compound eyes are significantly reduced in *smo*^{RNAi} animals but (R) not reduced in *ptc*^{RNAi} animals. Images of thoraces as well as heads are taken under the same magnification. cox, coxa; fem, femur; stl, sternellum; tib, tibia.

the ventral bristle patterning of the femur was disrupted and reduced, and the coxa was disproportionately shorter (Fig. 1A and B and Fig. S1). Moreover, legs in animals with severe knockdown

within the Hh signaling pathway in *Onthophagus* are likely conserved compared with those in other taxa, including *Drosophila*.

Functional Significance of Hh Signaling in the Development of Horns and Horn Polyphenisms. Paralleling the phenotypic effects described above, *hh*^{RNAi} and *smo*^{RNAi} resulted in similar horn phenotypes. However, neither horn location nor shape was affected in these animals; rather, *hh*^{RNAi} and *smo*^{RNAi} affected horn size. Furthermore, this effect was limited to males of small body size that had experienced suboptimal feeding conditions. Control-injected males, like WT males, only develop a full set of head horns when nutritional conditions allow them to reach or exceed a critical body size threshold (in this research, around 5.3 mm in pupal thorax width) (Fig. 3A). Male larvae that fail to reach this threshold size metamorphose into smaller, largely hornless morphs. However, almost all *smo*^{RNAi} males developed large head horns, including small individuals normally fated to remain hornless ($t_{31} = -5.80$; $P < 0.0001$). We observed a similar, albeit less extreme, effect in *hh*^{RNAi} males (Fig. 3A) ($t_{58} = -3.81$; $P = 0.0031$). The apparent linearization of the allometry among *smo*^{RNAi} animals precluded additional comparison with control-injected males. However, pairwise comparison of individual model parameters was possible between control-injected and *hh*^{RNAi} animals and found a highly significant difference in the allometric slope at the inflection point of the sigmoidal scaling relationship ($t_{73} = 2.75$; $P < 0.0001$) but not at any other model parameter (Fig. 3A).

Lastly, a partly corresponding effect was observed in pupal thoracic horns. Like all other species studied so far, *O. taurus* pupa develop a horn on the pronotum, which aids in the shedding of the larval head capsule during the larval to pupal molt (38). However, in a subset of species, including *O. taurus*, the pupal thoracic horn is fully resorbed before the adult molt (39). In WT as well as control-injected animals, this pupal thoracic horn scales linearly with body size, whereas *smo*^{RNAi} animals developed significantly longer horns relative to their body size compared with control-injected individuals (Fig. 3B) (ANCOVA on log-transformed data: $F_{\text{treatment}} = 115.88$; $P < 0.0001$; $F_{\text{treatment} \times \text{size}} = 98.97$; $P < 0.0001$). Similarly, although *hh*^{RNAi} males exhibited thoracic horn scaling relationships matching those of control-injected individuals for most of the body size range, males of intermediate body sizes around the body size threshold for head horns (~5.3 mm) had a tendency to also develop greatly exaggerated thoracic horns, resulting in significant differences in horn length residuals between control-injected and *hh*^{RNAi} males (Fig. 3B) (residual analysis: $t_{37} = -3.16$; $P = 0.0031$).

In contrast, *ptc*^{RNAi} resulted in a horn phenotype largely opposite to those generated by *hh*^{RNAi} and *smo*^{RNAi} (Fig. 1N and O). Note that, because *ptc*^{RNAi} also affected the prothorax to a degree that rendered it no longer useable for precise body size estimation, we were unable to quantify the exact relationship between horn length and body size. However, in all *ptc*^{RNAi} males ($n = 14$), not a single individual exhibited fully developed head horns regardless of overall body size, including in individuals of approximately similar or larger body size than their fully horned control-injected counterparts. Similarly, pupal prothoracic horns were reduced in size or in some cases, missing completely in both males and females (Fig. 2B). Taken together, our RNAi results, therefore, suggest that *hh* and *smo* inhibit horn development in small, low-nutrition males, whereas *ptc* promotes horn formation, at least in high-nutrition individuals.

Developmental Evolution and Integration of Nutrition Sensitivity, Allometry, and Threshold Sizes. Signal transduction pathways have been proposed to constitute developmental/genetic modules that, on one side, maintain a high level of conservation within, whereas on the other side, are easily dissociated from their original biological process and co-opted into novel developmental contexts, where they can then facilitate the evolution of novel traits and trait

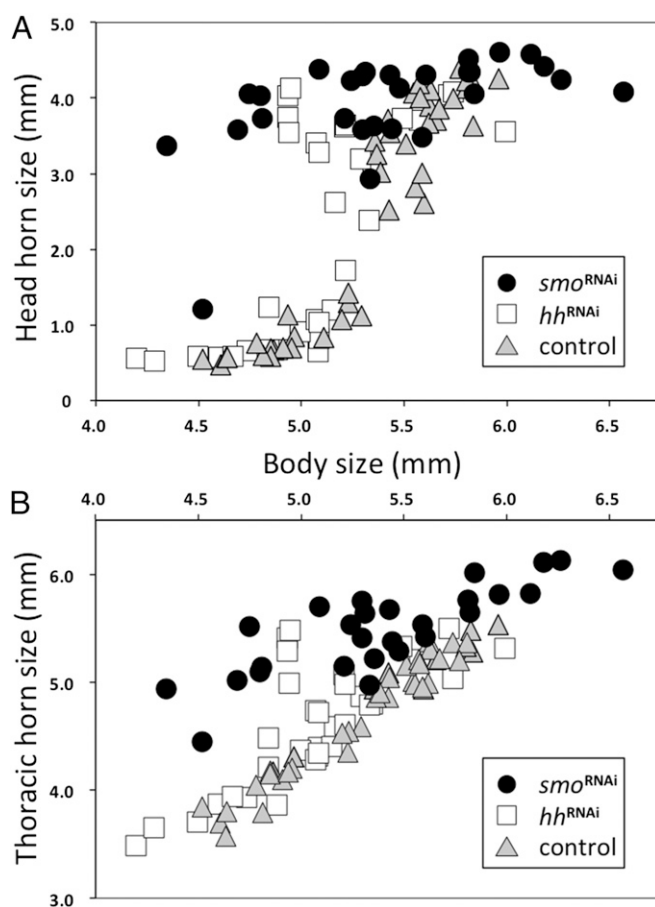


Fig. 3. Effect of *hh*^{RNAi} and *smo*^{RNAi} on horn development and relative horn sizes in male *O. taurus* pupae. (A) Control-injected animals (gray triangles) exhibited the species-typical sigmoidal relationship between body size (x axis) and head horn size (y axis); *smo*^{RNAi} (●), in contrast, resulted in nearly all males developing full-sized horns regardless of body size and a complete linearization of the body size horn length allometry. Lastly, *hh*^{RNAi} (□) also resulted in relatively longer horns, but this effect was limited to a subset of males of intermediate body size. (B) *hh*^{RNAi} (□) modestly increased thoracic horn length in some animals, whereas *smo*^{RNAi} (●) increased horn length in all individuals, especially small, low-nutrition individuals.

characteristics (40). Previous work suggests that polyphenic, sigmoidal relationship between body size and horn size evolved from linear and isometric scaling relationships (16). Recent work has implicated the sex determination gene *dsx* in promoting horn formation in males subject to optimal nutritional condition in both the rhinoceros beetle *Trypoxylus dichotomus* (which exhibits a linear, highly positive allometry) and *O. taurus* (which exhibits a more derived sigmoidal allometry, including a sharp body size threshold separating alternative male morphs). In both taxa, *dsx* seems to play a critical role in the nutrition-dependent exaggeration of horn growth, and several studies suggest further that insulin signaling, juvenile hormone signaling, or a combination of the two may interact with *dsx* and sensitize *dsx* action to nutritional conditions (24, 25, 27). This research highlights a third highly conserved but previously overlooked pathway, the Hh signaling pathway, which—like *dsx* signaling—has been co-opted into the regulation of horn development and acquired nutrition sensitivity in the process but exerts its regulatory function in, to the best of our knowledge, a thus far unprecedented manner, namely by actively inhibiting horn growth in low-nutrition individuals only (Fig. 4A).

Intriguingly, these findings raise the possibility that a combination of *dsx*-mediated promotion of horn growth under high nutrition

dependent variable, treatment as a fixed factor, thorax width (body size) as a covariate, and treatment \times thorax width as an interaction term. We first executed this analysis by including all three treatment groups and repeated it for the raw as well as log-transformed data, but we discovered significant differences in error variance among samples with both approaches. We then executed the same analysis in pairwise comparisons. Here, analysis of log-transformed data permitted pairwise comparisons between control-injected and smo^{RNAi} as well as between hh^{RNAi} and smo^{RNAi} males, respectively, but not between control-injected and hh^{RNAi} males. Lastly, we also replicated the residual-based analysis executed for head horns for thoracic horns. We used a simple linear regression to model the allometry of control-injected males and calculated residuals as the difference between horn lengths observed in a given

individual and those expected for a given individual's body size based on this regression. Two-tailed t tests were used for pairwise comparisons. For simplicity, figures show raw data only. Results from t tests are presented as t_{df} = test statistic.

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