



Gene regulatory networks underlying the development and evolution of plasticity in horned beetles

Phillip L Davidson^{1,2}, Erica M Nadolski^{1,3} and Armin P Moczek⁴

Horned beetles have emerged as a powerful study system with which to investigate the developmental mechanisms underlying environment-responsive development and its evolution. We begin by reviewing key advances in our understanding of the diverse roles played by transcription factors, endocrine regulators, and signal transduction pathways in the regulation of horned beetle plasticity. We then explore recent efforts aimed at understanding how such condition-specific expression may be regulated in the first place, as well as how the differential expression of master regulators may instruct conditional expression of downstream target genes. Here, we focus on the significance of chromatin remodeling as a powerful but thus far understudied mechanism able to facilitate trait-, sex-, and species-specific responses to environmental conditions.

Address

Department of Biology, Indiana University Bloomington, IN 47405-7107, United States

Corresponding author: Moczek, Armin P (armin@iu.edu)

¹ Shared co-first authors.

² ORCID: 0000-0003-0414-2957

³ ORCID: 0000-0003-3314-5305

⁴ ORCID: 0000-0002-3478-9949

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Introduction

Developmental plasticity enables organisms to adjust components of their phenotype in response to changes in the environment, often in an adaptive manner [1]. Developmental (or phenotypic) plasticity is taxonomically widespread and manifests on every level of biological organization, from differential gene expression and hormone physiology to behavior [2]. Further, developmental plasticity shapes evolutionary trajectories of

natural populations by buffering organisms against environmental perturbations, providing important targets for selection, and biasing phenotypic variation visible to evolutionary processes [3]. The mechanisms and consequences of plasticity have received particular attention in insects, including plasticity's contribution to biodiversity (e.g. ants [4]), ecosystem services (e.g. dung beetles [5,6]), and the impact of agricultural pests (e.g. aphids [7], planthoppers [8]). Here, we review recent findings on the mechanisms and evolution of developmental plasticity in horned beetles in the genus *Onthophagus*, an emerging model system in ecological and evolutionary developmental biology, and synthesize with findings in related taxa.

Onthophagus are true dung beetles, that is, both larvae and adults consume dung as food sources. While *Onthophagus* species have radiated onto an amazing diversity of dung types across all continents save Antarctica [9], most species used for research generally utilize the dung of large herbivorous mammals, and in particular that of cattle, and are thus easily maintained and reared in the laboratory [10]. Horned beetles have attracted the attention of plasticity researchers primarily because of their pronounced condition-dependent development, morphology, and behavior [6]. *Onthophagus* reproduces by constructing underground *brood balls* out of dung into which females deposit a single egg. Larvae hatch and sustain their entire growth and subsequent metamorphosis from resources extracted from feeding on this brood ball. Because of natural variation in brood ball size, quality, and ecological circumstances of oviposition, eclosing adults exhibit a wide range of body sizes [5,10,11]. Male adults compete aggressively with each other over access to females, and in many species, large males develop exaggerated horns on their head, thorax, or both, which function as effective weapons in male combat [12]. Smaller-sized males, however, are inferior fighters given their size and in many species do not invest in horns and instead engage primarily in nonaggressive sneaking behaviors and sperm competition, including enlarged testes and ejaculate volumes [13]. In a subset of species, nutrition-responsive development is especially pronounced and has given rise to alternative *major* (horned) and *minor* (hornless) male morphs so distinct that some have originally been described as different species [14]. In partial contrast, female *Onthophagus* typically exhibit a similar range of

adult body sizes yet very rarely develops horns. While morphological diversity within males is driven by nutritional variation during larval development, sexual dimorphism is the result of sex-specific development following XX/XY sex determination [15]. As described below, however, both forms of conditional development share important developmental and genetic mechanisms.

Diverse developmental mechanisms facilitate conditional development in horned beetles

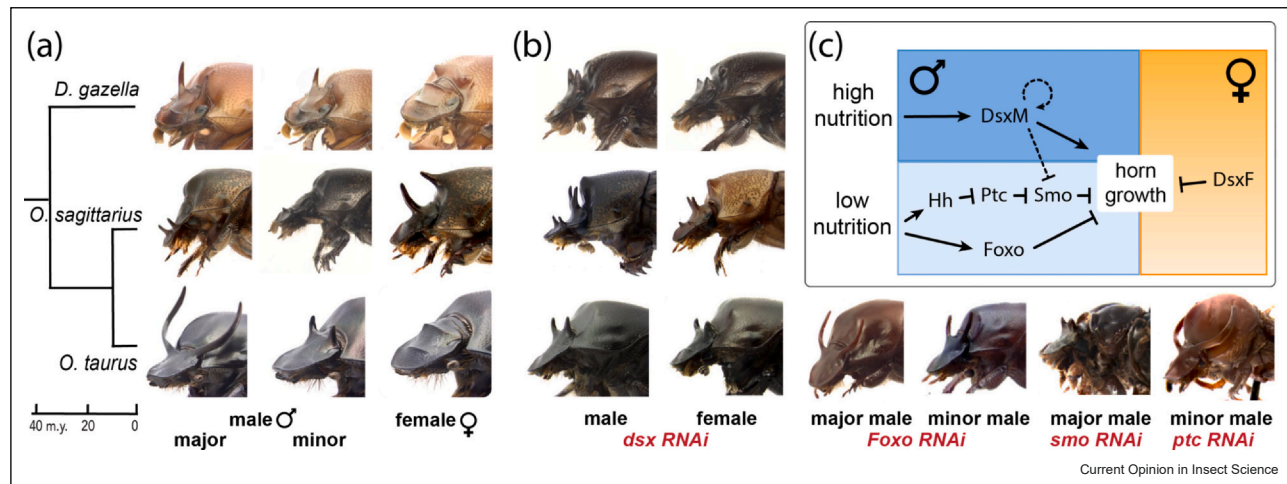
A key pathway in coordinating nutrition-dependent growth across vertebrate and invertebrate taxa is the insulin/IGF signaling (IIS) pathway. Work across a number of species also documents the prominent role this pathway plays in regulating nutritional plasticity in horned beetles, yet at the same time highlights the evolutionary lability of pathway components in trait development. For example, RNAi-mediated downregulation of Forkhead-box O (Foxo), a transcription factor best known as a growth inhibitor in low-nutrition conditions, demonstrated FoxoRNAi modestly increased male horn length in the horn-polyphenic *O. nigriventris*, especially under high-nutrition conditions [16]. Subsequent work in *O. taurus* [17] and *Digitonthophagus gazella* [18] also documented an increase in horn length following FoxoRNAi, but did so in low-nutrition males only, whereas high-nutrition males exhibited a modest horn length reduction, thereby effectively linearizing the normally sigmoidal body size–horn length allometry in both species. In contrast, knockdown of the insulin receptors InR1 and InR2 separately or in combination failed to affect horn size in either *O. taurus* or *D. gazella*. Interesting patterns were also observed for other morphological traits. For example, FoxoRNAi increased male copulatory organ size in *O. nigriventris* [16] and *D. gazella* [18] but decreased it in *O. taurus* [17]. Likewise, RNAi targeting InR1 and InR2 yielded partly divergent effects on genitalia scaling in *O. taurus* and *D. gazella* [17,18]. Taken together, these results suggest that while the IIS pathway has maintained a general function in linking larval nutrition to growth in horned beetles, different pathway components may diverge rapidly in their specific function across different body regions and species. In contrast, the potential functional roles played by insulin-like peptides (ILPs), the receptor-binding ligands of the IIS pathway, have yet to be investigated in *Onthophagus*, but work emerging in *Drosophila* indicates that different ILPs may functionally diverge to differentially regulate body regions and developmental processes in response to both nutrition and immune challenges [19,20], indicating a promising avenue for future research on the regulation and evolution of plasticity in beetles.

Important additional insights emerged from investigations of the *hedgehog* (Hh) signaling pathway [21], another highly conserved pathway best understood for its role in patterning anterior/posterior (A/P) polarity of diverse

traits. Work to date has targeted the Hh ligand — a diffusible morphogen required for activation of the pathway, Patched (*ptc*) — the membrane-bound Hh receptor, and Smoothened (*smo*) — a membrane protein that is bound and sequestered by Ptc until the receptor instead binds Hh, leaving Smo to activate downstream intracellular signaling. RNAi phenotypes for these genes demonstrated on one side that interactions among pathway members are conserved in *O. taurus* development, including the regulation of A/P polarity in appendages. However, the same work documented a novel role of Hh signaling in the regulation of nutrition-responsive horn formation: inhibition of Hh signaling by *hh*^{RNAi} or *smo*^{RNAi} led to development of large horns even in low-nutrition males, whereas constitutive activation of the pathway by *ptc*^{RNAi} eliminated horn formation even in the largest males. Combined, these findings indicate that at least in *O. taurus*, Hh signaling selectively suppresses horn formation in low-nutrition males only, however, the function of Hh signaling in other horned beetle taxa remains to be investigated [21].

Similarly significant was the implication of the sex-determination factor *doublesex* (*dsx*) in regulating not only the sex-limited expression of horns, but also their nutritional plasticity [15]: specifically, *dsx*^{RNAi} was found to eliminate both the sex-specificity of horn induction and the dramatic polyphenism in these horns by simultaneously promoting the growth of small horns in females of all body sizes while inhibiting the growth of exaggerated horns in large males, yielding sexually monomorphic individuals regardless of sex and body size. The resulting sexual monomorphism was mirrored in other normally dimorphic body regions, including foretibiae and genitalia, but the effect on allometry was both surprising and unique to horns. These findings have now been replicated in a second species, *D. gazella* [22]. Kijimoto et al. [15] also investigated the potential divergence in *dsx* function across species by performing additional knockdowns in *O. sagittarius*, an unusual species in which females produce exaggerated posterior head and prothoracic horns, whereas males only develop a pair of modest, anterior head horns. *dsx*^{RNAi} in female *O. sagittarius* reduced prothoracic horn size, induced ectopic paired anterior head horns, and led to the striking formation of a branching posterior head horn. *dsx*^{RNAi} in males promoted growth of both an ectopic prothoracic horn and induced a branching posterior head horn, but had no effect on anterior head horns, again leading to the production of sexually monomorphic individuals. Combined, these results indicate that rapid evolution of *dsx* function in dung beetles underlies the diversification of morphological development conditional upon sex and nutrition, including the evolution of both exaggerated polyphenisms and reversed sexual dimorphism. Recent work now suggests that at least some of this evolutionary lability may be enabled by DSX

Figure 1



Molecular mechanisms of conditional development in horned dung beetles. (a) Representative nutritionally plastic and sexually dimorphic horn phenotypes in three species in the tribe Onthophagini. (b) RNAi phenotypes implicating signaling pathways in the regulation of horn expression: sex determination — *dsx* [15,22], insulin (IIS) signaling — *Foxo* [17], and Hh signaling — *smo*, *ptc* [21]. (c) Proposed model for the regulation of nutritionally plastic and sexually dimorphic development of horns in *Onthophagus taurus*. Diverse pathways contribute to the nutrition-dependent expression of alternate male morphs and link the regulation of sex determination to nutritional plasticity. Current data suggest that the Hh signaling pathway negatively regulates horn growth in low-nutrition males. This process is also potentially regulated by the IIS pathway, which appears to repress horn growth in low nutrition conditions via activation of the growth inhibitor Foxo. Horn growth under high-nutrition conditions in turn is promoted by the male-specific isoform of the sex-determination factor *dsx*, while the female-specific isoform(s) repress horn formation in female *O. taurus*. Data from Dsx-binding site analyses and RNA sequencing [24] suggest the possibility that Dsx may negatively regulate *smo* in head horn tissue from large males while positively regulating its own locus, suggesting a potential link between these two pathways.

isoform-specific target gene repertoires, which may diverge further as a function of trait and likely species [23–25]. More generally, these results can now be used to develop models and motivate future investigations regarding how different regulators of conditional development may interact, and how conditional development may diversify (Figure 1).

Many of the plasticity regulators identified in *Onthophagus* have now also been implicated in condition-dependent development in other insect taxa. For example, sex-specific splicing of *dsx* isoforms has been found to facilitate sex-specific mandible growth in the stag beetle, *Cyclommatus metallifer* [26] and sex-specific head and thoracic horn growth in the rhinoceros beetle *Trypoxylus dichotomus* [27,28]. Aside from IIS signaling being repeatedly co-opted during the independent evolution of eusociality in bees, wasps, ants, and termites — another rich example of polyphenism in insects [29] — the insulin receptor InR has also been implicated in the regulation of plasticity of male horns in the rhino beetle [30]. In addition, early hormone application studies using the juvenile hormone (JH) analog methoprene also supported a role for JH in the regulation of horn development and plasticity in beetles [31–34], and more recent work on mandible exaggeration in stag beetles [26] and *Gnathoceros* flour beetles [35] using the

same topical application approach raised the possibility that JH may promote trait exaggeration more broadly. However, as discussed in detail in Zera (2007) [36], topical hormone applications are — especially if used as the sole mode of investigation — prone to generate misleading outcomes, for instance, through the cross-stimulation of other pathways due to excessive dosages [36]. It is worth noting here that methoprene-treated *Onthophagus* generally failed to survive high-dosage treatment and instead nearly invariably died during the larval-to-pupal molt, consistent with the possibility that the observed phenotypes may simply reflect artifactual nontarget outcomes [31–33]. Importantly, no additional work has been carried out in *Onthophagus* beetles or other taxa that would independently support a functional role of JH in horn polyphenism. Interestingly, this lack of support parallels the direction of discoveries in other insect plasticity contexts, including wing polyphenisms in hemimetabola (solitary/winged gregarious morphs in crickets [37], winged dispersal morphs in aphids and planthoppers [8,38]).

Regulation and transduction of condition-dependent expression

Condition-specific transcriptional regulation is dependent first on regulatory molecule (e.g. transcription factor) availability in the nuclear environment, as the

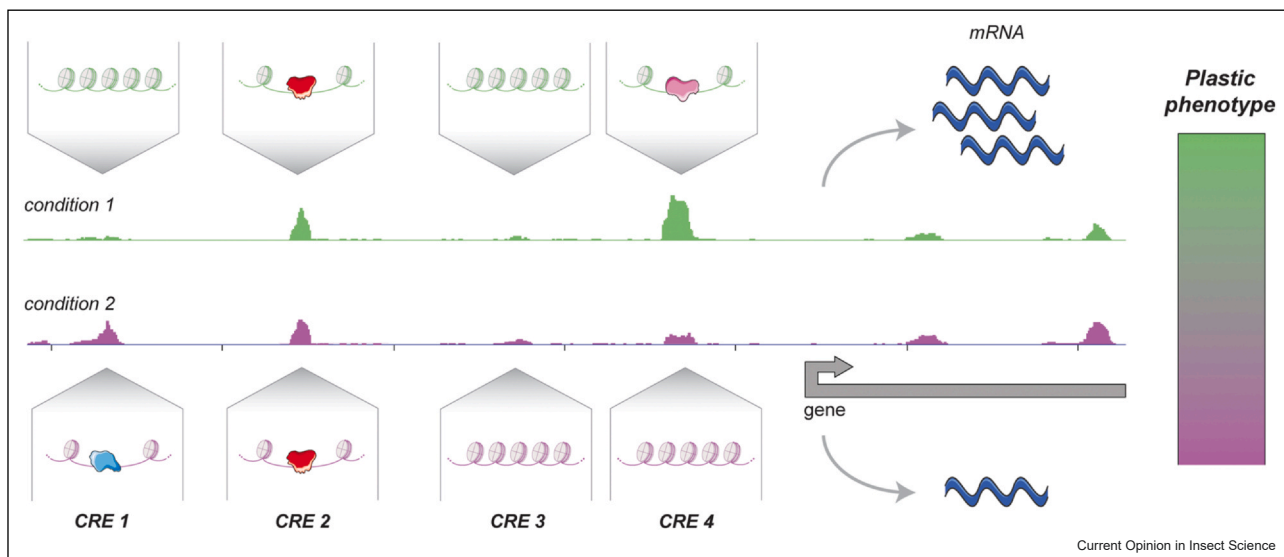
binding of regulatory factors to cofactors and *cis*-regulatory elements (CREs) can induce gene expression change. As highlighted above, many of these genes and pathways whose expression underlies plasticity in horned beetle development have now been characterized. However, the regulatory mechanisms *establishing* such condition-specific expression in the first place are poorly understood. Likewise, how such conditional expression — once established — is subsequently transduced across developmental gene networks to generate alternative phenotypes remains essentially unknown. Regulatory elements are likely central to these unresolved questions [39], as they constitute noncoding genomic elements that connect molecular networks via the binding of regulatory factors, which subsequently induces changes in gene expression. Thus, in addition to measuring gene expression levels, characterizing the role of regulatory elements in natural systems will afford a more mechanistic understanding of the evolution and development of condition-dependent trait development.

Two critical molecular properties that can determine the function of regulatory elements are their *sequence* and *accessibility*. Regulatory element sequence defines the binding affinity of different transcription factors to the locus, but this property is *not* condition-dependent as regulatory element sequence remains constant over an organism's lifetime (barring somatic mutation) and

across developmental contexts. Accessibility, on the other hand, relates the binding capacity of transcription factors to a regulatory element via differences in chromatin conformation, that is, 'open' chromatin permits transcription factor binding, whereas 'closed' chromatin obstructs it. Unlike its nucleotide sequence, the accessibility of a regulatory element is highly context-dependent and may vary over developmental time and across cell types in response to molecular activities such as histone modifications by chromatin-modifying enzymes or binding of pioneer factors [40]. Thus, regulatory elements are predicted to play an important role in the regulation and evolution of developmental plasticity (Figure 2), though their functions in natural systems are largely undescribed.

Historically, research in these areas in horned beetles and other nontraditional model systems has been hindered by a lack of high-quality genomic references, which is essential for linking regulatory element activity to changes in gene expression. However, recent technological advances enabling the sequencing and assembly of chromosome-scale genomes are opening new research avenues for examining regulatory mechanisms underlying phenotypic plasticity in a wide variety of organisms. For example, genome-wide measurements of transcription factor binding and chromatin accessibility using ChIP-seq [41] and ATAC-seq [42], respectively,

Figure 2



Model of *cis*-regulatory control of condition-dependent chromatin accessibility. Variable environmental conditions induce phenotypic responses of plastic traits via changes in gene expression. These changes in gene expression are controlled in part by differential availability and combinatorial binding of regulatory factors to CREs, which is dependent on (among others) two molecular properties of the CRE: 1) its sequence and 2) accessibility. In this model, alternative environmental conditions (green and purple) induce changes in chromatin accessibility of CREs regulating a nearby gene, altering the profile of transcription factor binding at this locus, and generating downstream transcriptional changes associated with phenotypic plasticity. High-throughput sequencing assays such as ATAC-seq, which estimates chromatin accessibility and the location of putative CREs at a genome-wide scale, show great promise for characterizing the *cis*-regulatory basis for plastic gene regulatory changes in diverse insect systems.

can quickly estimate the location (and in ATAC-seq, accessibility) of putative regulatory elements for a certain tissue. ATAC-seq is an especially attractive option given its relatively low tissue input amount (~50 000 cells), a consideration particularly relevant for small or difficult-to-obtain specimens. Multi-omic approaches (e.g. ATAC- or ChIP-seq paired with RNA-seq) can be especially powerful for characterizing transcriptional dynamics in a tissue type, as this combined approach can quickly profile genome-wide regulatory mechanisms and expression with little-to-no prior information of the system. For example, recent work in honey bees has demonstrated unique regulatory architectures in the brains of queens, drones, and workers, potentially associated with alternative behavioral phenotypes observed across honey bee sexes and castes [43].

Within horned beetles, early attempts to investigate the role of histone modifications in nutrition-responsive development have detected at least some associations between chromatin-modifying enzymes and trait plasticity. For example, Snell-Rood and colleagues [44] reported that methylation patterns in *O. taurus* vary with developmental nutrition at a subset of genomic loci. Furthermore, knockdown of histone deacetylase-3 (HDAC3) expression via RNAi reduced horn size and altered horn shape in the same species [45,46], likely due to changes in downstream regulation of horn network genes. Similarly, HDAC3^{RNAi} exaggerated — while HDAC1^{RNAi} reduced — nutrition-sensitive mandible formation in *Gnathoceros* [47]. Overall, these results are concordant with the idea that divergent regulatory element activity underlies the development of plastic trait formation, as histone modifications including methylation and acetylation result in chromatin configuration changes and by extension, variable accessibility of DNA to transcription factor binding. Recent work has therefore sought to profile genome-wide chromatin accessibility patterns in developing beetle horns and begun to identify distinct regulatory architectures underlying the modulation of condition-dependent horn phenotypes, including an enrichment of binding motifs of critical developmental transcription factors at sex- and nutrition-responsive regulatory elements. Collectively, these results suggest regulatory elements play a prominent role in mediating developmental plasticity and begin to provide a much more mechanistic understanding of the developmental regulation and diversification of plasticity, in ways that may be broadly applicable to insects and beyond.

Next frontiers: evolution of chromatin remodeling and conditional gene regulatory networks (GRNs)

Organisms can be thought of as mosaics of traits that vary in the degree to which they develop in a condition-dependent manner in response to external and internal

stimuli [48]. For example, as discussed above, horn shape and size are generally highly sensitive to larval nutrition and sex in *Onthophagus*, whereas wing development is not and instead varies primarily as a function of overall body size. This diversity of condition-dependence among traits within an organism reflects adaptive divergences in response to selection favoring different sensitivities to developmental and/or environmental circumstances (e.g. somatic sex, nutrition availability, infection state, and population density) and the capacity of plasticity to evolve given a trait's underlying genetic architecture [1,3,49]. Even though environment-sensitive development has been studied extensively in diverse systems and several key regulators of condition-dependent trait formation have been identified, our understanding of the gene regulatory mechanisms underlying condition-sensitive development, as well as how those may diversify across traits, species, and conditions, remains largely incomplete. Horned beetles are no exception; many of the regulatory pathways implicated as regulators of nutrition-sensitive development in *Onthophagus* have been studied in single traits and species only, leaving largely unaddressed how these processes may be adjusted as a function of trait type within the same individual organism. Similarly, how condition-responsive development diversifies among species or populations is largely unknown. Given *Onthophagus*' species richness, history of introductions as part of biocontrol measures, and recent range expansions, many opportunities exist to address these and related questions over a range of phylogenetic distances [6,50].

Such research may also be able to shed light on fundamental questions regarding GRN evolution. Condition-dependent traits such as those involved in nutrition responsiveness and sexual dimorphism are among the fastest-evolving phenotypic classes, yet it is largely unknown if the same gene networks involved in mediating condition-dependence of a given trait in a single species also mediate diversification of this trait across species or — alternatively — whether trait formation on one side and context responsiveness on the other are developmentally and evolutionarily decoupled. Increasing availability and affordability of accurate, contiguous reference genomes shows great promise for beginning to tackle these questions. Regulatory element properties such as sequence and accessibility (discussed above) can be compared between species to identify how the binding capacity of distinct transcription factors to regulatory elements may have evolved. Furthermore, comparative genomic assays can identify gains or losses of entire regulatory elements, another way regulatory interactions within GRNs can evolve. Taken together, comparing patterns of regulatory evolution underlying trait plasticity and development within species to those underlying between species variation provides exciting

opportunities to better understand the evolutionary lability and pleiotropic constraints shaping gene regulation and organismal diversification.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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