Development and evolution of insect polyphenisms: novel insights through the study of sex determination mechanisms

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Polyphenism is defined as a single individual's ability to develop into two or more alternative phenotypes. Polyphenic development is taxonomically widespread among insects, cued by diverse environmental factors, and enables single genotypes to accommodate breathtaking phenotypic diversity. Most research on the developmental regulation and evolution of insect polyphenisms has focused on endocrine control mechanisms, in particular the role of the sesquiterpenoid juvenile hormone. Here we review recent finding that suggest additional and previously overlooked mechanisms that underlie the developmental regulation and rapid evolution of polyphenic development. Specifically, we focus on the role of somatic sex determination mechanisms, which mediate body-region and tissue-specific differentiation as a function of sex across insects. Recent work on *Onthophagus* horned beetles suggest that the same mechanisms have been co-opted to regulate the development of nutritionally cued, alternative male morphs, and that rapid changes in these mechanisms underlie rapid evolutionary changes in patterns of phenotype expression across *Onthophagus* species, including the loss of old and gain of novel locations for horn development, the evolution of reversed sexual dimorphisms, and the secondary loss of male polyphenism. We discuss how these findings expand the way we think about the origins and diversification of polyphenisms, and close by briefly highlighting potentially fruitful avenues for future research.

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Introduction — a brief primer on insect polyphenisms

Polyphenic development enables a single individual or genotype to give rise to two or more discrete phenotypes depending on environmental conditions [1]. Polyphenic development is found across phyla, and reaches some of its most extreme manifestations within the insects [2]. Well-known examples include seasonal polyphenisms in the Lepidoptera, phase polyphenisms in locusts, alternations between sexual and asexual reproductive modes in aphids, castes in social insects, wing polyphenisms in crickets or alternative fighter and sneaker morphs in many beetles (reviewed in [1,3]). Polyphenic development can be responsive to a wide range of cues, as well as combinations thereof, from photoperiod and nutrition to social conditions and host plant age [4]. Lastly, polyphenic development entails the coordinated, and typically adaptive, divergence of syndromes of traits: for example, social insect castes not only differ in size and shape, but also in relative investment into reproductive structures, energy metabolism, and behavioral repertoire [5,6]; likewise, seasonal butterfly morphs diverge not just in wing patterns, but also in wing shape, flight and hiding behavior, and mate preferences [1,7,8]. As such, polyphenic development enables individuals to develop into strikingly different, yet highly functionally integrated, phenotypes to suit diverse ecological conditions.

Most research on the developmental regulation and evolution of insect polyphenisms has focused on endocrine control mechanisms, in particular the role of the sesquiterpenoid juvenile hormone (JH) [9–11]. Juvenile hormone plays a fundamental role in the regulation of metamorphosis, where its presence or absence during nymphal and larval instars determines whether developing animals molt into the same developmental stage (e.g. larva-to-larva) or transition to the next stage (e.g. larva-to-pupa, or pupa-to-adult), respectively, a capacity that has earned JH the synonym status quo hormone [9]. Numerous studies have now well established that aspects of JH signaling have been co-opted into the regulation of polyphenic development (reviewed in [4,11]) and that evolutionary changes in JH metabolism and ontogenetic timing of tissue sensitivity to circulating JH underlie evolved changes in polyphenic development in artificially selected or naturally evolved populations and species (e.g. [12–15]). Very recent studies on polyphenic beetles now suggest that somatic sex-determination mechanisms have undergone a partly parallel evolutionary journey: from regulating basic and fundamental aspects of insect development to their secondary co-option into the context of polyphenic differentiation [16•,17•].

Somatic sex-determination in insects — the role of doublesex

In insects, the sex determination pathway regulates the sex-biased expression of downstream target genes, which
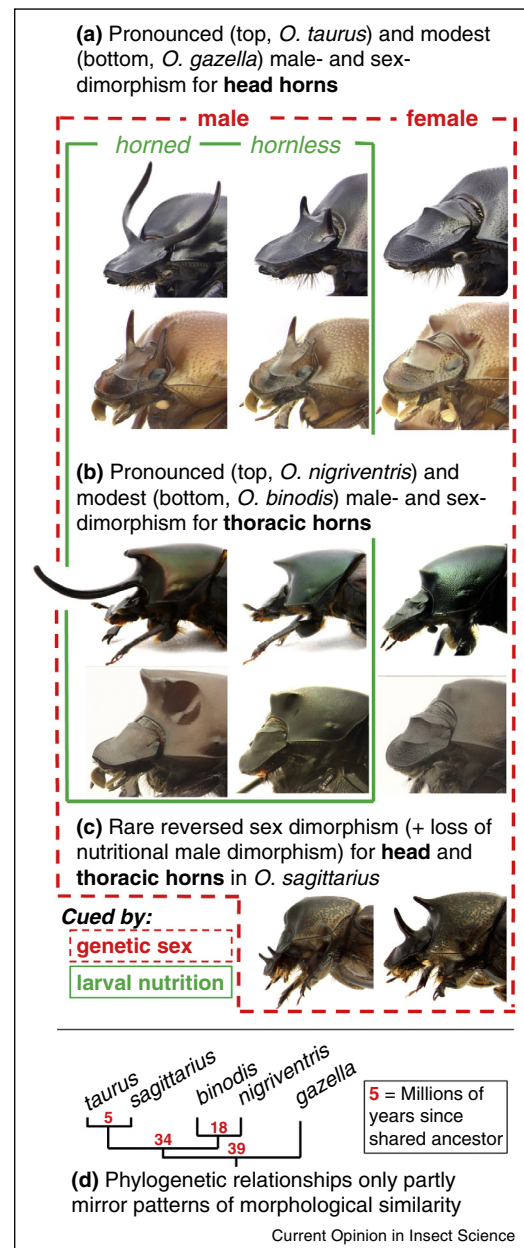
in turn facilitates the expression of sex-specific development and behavior across diverse insects [18–20]. The transcription factor Doublesex (Dsx) plays a particularly significant role in this pathway, which is best understood from studies in *Drosophila*. In *D. melanogaster*, a hierarchy of sex-determination genes acts to regulate the expression of male- and female-specific splice variants of *dsx*, which in turn regulate the sex-biased expression of downstream target genes responsible for the elaboration of sexually dimorphic traits [21]. Even though the sex-determination pathway *upstream* of *dsx* is surprisingly divergent across insect orders, *dsx* itself is highly conserved, including most notably the expression of male- and female-specific transcription factors generated via alternative splicing [19,20].

Furthermore, recent work has shown that *dsx* is a nexus for the evolution and diversification of sexually dimorphic traits, either via changes in *cis*-regulatory sequences of *dsx* target genes [22,23] or changes in the expression of *dsx* itself [24,25]. Additionally, the recognition that developing *Drosophila*, and likely most insects in general, are mosaic for *dsx* expression, and thus are mosaic for the potential to differentiate sexually, has led to a greater appreciation for the potential of evolutionary diversification of sexual dimorphisms through tissue- and body region-specific changes in *dsx* activity [26]. Recent studies on horn-polyphenic beetles now suggest that *dsx*-mediated regulation of development has been recruited into the regulation of *nutrition*-specific differentiation of alternative horned and hornless fighter and sneaker morphs.

Sex- and morph-specific development in horn-polyphenic beetles may be regulated via differential expression of sex-specific Doublesex isoforms

Horn and horn-like structures have evolved in at least seven beetle families, where they function as weapons of sexual selection (reviewed in [27]). Horn development is highly variable within and between sexes as well as among species, and is heavily influenced by both genetic (e.g. sex) and environmental factors (e.g. nutrition; Figure 1). For instance, in nearly all species horn development is restricted to males and absent in females [28]. Female hornlessness arises as a consequence of canalized sex-specific development, most likely following XX/XY sex determination (males are XY, e.g. [29]) and is not affected by nutritional conditions: in these cases all females, regardless of larval nutrition and resulting adult body size, are hornless. In contrast, horn development in males is not nearly as canalized, and is frequently influenced by nutritional conditions (e.g. [30–32]). In many species, variation in larval nutrition cues the expression of distinct, alternative horned (large) and hornless (small) morphs among males. In these cases, male phenotype is the result of developmental plasticity: all males have the capacity to develop into either morph, but choose one or

Figure 1

(a) Pronounced (top, *O. taurus*) and modest (bottom, *O. gazella*) male- and sex-dimorphism for **head horns**

(b) Pronounced (top, *O. nigriventris*) and modest (bottom, *O. binodis*) male- and sex-dimorphism for **thoracic horns**

(c) Rare reversed sex dimorphism (+ loss of nutritional male dimorphism) for **head** and **thoracic horns** in *O. sagittarius*

Cued by:
genetic sex
larval nutrition

(d) Phylogenetic relationships only partly mirror patterns of morphological similarity

5 = Millions of years since shared ancestor

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Phenotypic diversity among five *Onthophagus* species and their phylogenetic relationships. **(a)**–**(c)** Diversity in sexual-dimorphisms (red box, cued by genetic sex) and male-dimorphisms (green box, cued by nutrition). **(d)** Phylogenetic relationships among the same five species, which only partly mirror patterns of morphological similarity [35].

the other pathway depending on feeding conditions encountered during larval development. Importantly, the resulting alternative morphs differ not only in the presence of elaborate horns, but in a whole syndrome of co-diverging morphological, physiological, and behavioral phenotypes, ranging from reproductive tactics, aggression and paternal investment to testes development and thermoregulatory behavior [16•,27]. Lastly, species have

Figure 2
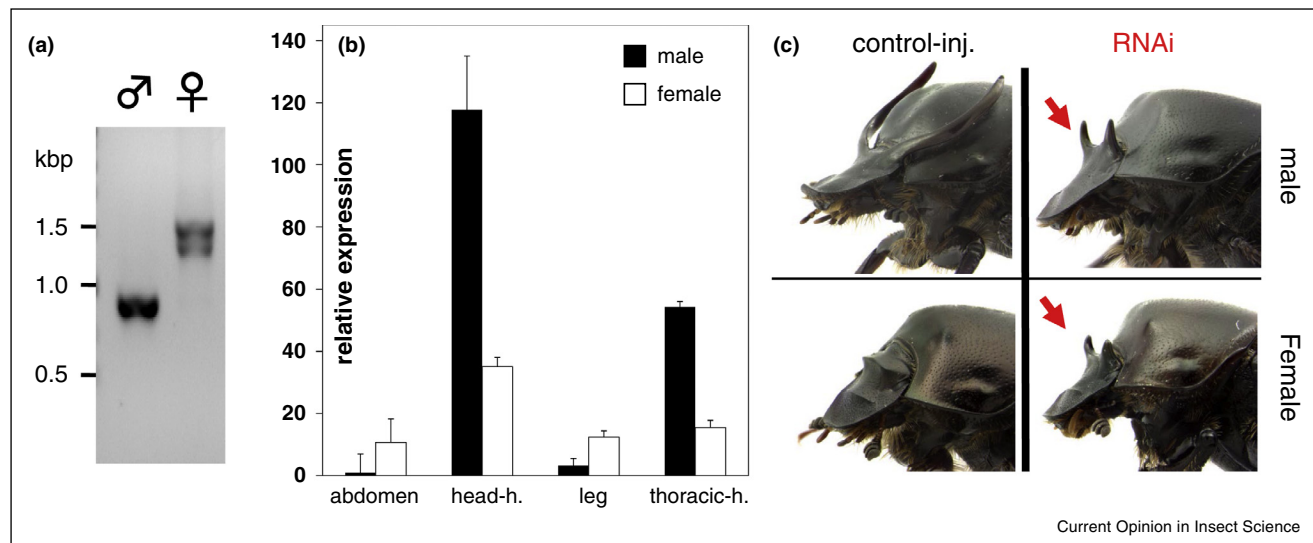
(a) *dsx* is expressed in alternative male-specific and female-specific splice variants. (b) *dsx* expression is elevated in the horn tissue of large males relative to their legs or abdominal epidermis or any corresponding tissue in large females. (c) *dsx*RNAi greatly reduces horns in large males (top) but induces horns in large females (bottom; modified after [16•]).

diversified greatly in the nature and degree of both sexual- and male dimorphisms as well as the interactions between the two, suggesting that both developmental contexts significantly enriched the evolutionary degrees of freedom available to horned beetle lineages [28].

Early microarray experiments designed to contrast gene expression in developing males and females of the horn-polyphenic beetle *Onthophagus taurus* (Figure 1a) detected high levels of transcript abundance of the transcription factor Doublesex (Dsx) in developing head-horns and thoracic horns in large males but not females [33,34]. Subsequent quantitative and functional analyses yielded three major insights (Figure 2; [16•]): firstly, *dsx* expression is conserved: as in other insects, male- and female-specific isoforms are expressed in a strictly sex-specific manner via alternative, sex-specific splicing (Figure 2a). Secondly, individuals are mosaics of body regions that differ in the degree to which they are sexually differentiated, with the level of *dsx*-expression correlating positively with the degree of sex-specific growth differences across body regions (Figure 2b). Finally, when *dsx* transcript abundance was diminished via larval RNAinterference (RNAi) this greatly reduced horn development in males but *induced* conspicuous ectopic head horns in females (Figure 2c). These results suggested that much like in *Drosophila*, differential expression of male- and female-specific Dsx isoforms facilitates body region- and tissue-specific differentiation between males and females. When *dsx* is knocked down, both sexes converge onto an intermediate form.

Remarkably, *dsx*-expression also revealed conspicuous variation depending not just on body region and sex, but also as a function of nutrition. More detailed analyses of the interactions between nutrition and *dsx* expression and function yielded the following results: firstly, in *O. taurus*, expression levels of the male splice variant *increased* with nutrition, but *only* in body regions showing disproportionate growth responses to increased nutrition (Figure 3a); secondly, *dsx* knockdown in males disproportionately affected horn development in large, high-nutrition males. Small-horned and medium-horned males showed no or only moderate reductions in horn size, whereas as high-nutrition and fully horned males lost in excess of 80% of their horn length ([16•]; Figure S7). In contrast, finally, *dsx*RNAi in females not only *induced* horns, but did so in a *nutrition-dependent manner*, with large, high-nutrition females developing relatively greater horns than low-nutrition females (Figure 3b). These data support the hypothesis that *dsx* not only mediates development of sex-specific traits, but may have been coopted to facilitate the development of nutritionally cued phenotypes within sexes [16•].

Evolutionary changes in doublesex function underlie rapid divergence in sex and morph-specific development in polyphenic beetles

Onthophagus species have diverged in a variety of ways in patterns of sex-specific and morph-specific development (Figure 1). An especially extreme case involves *O. sagittarius* (Figure 1c), which among the five species depicted in Figure 1 is most closely related to *O. taurus* [28,35], yet
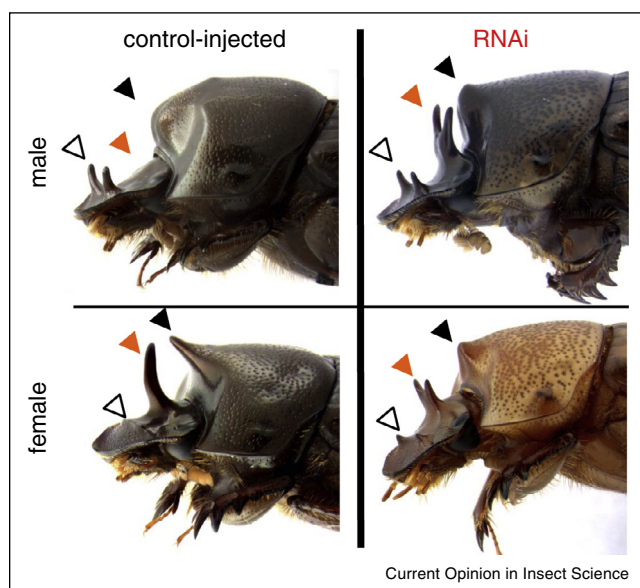
Figure 3
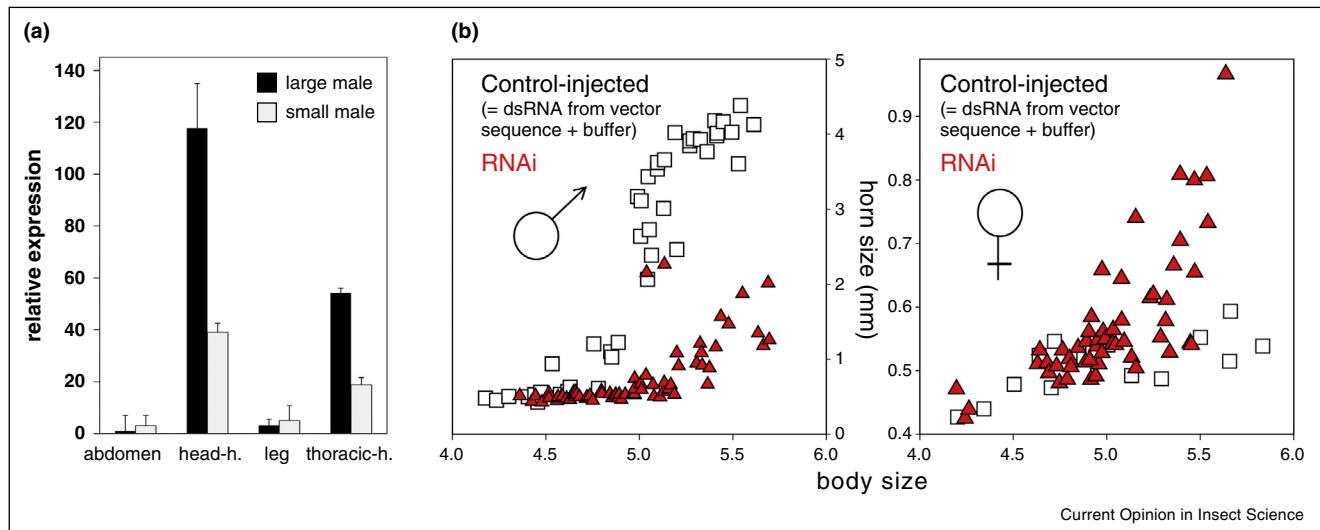
(a) *dsx* is highly expressed in male traits that exhibit an elaborate growth response to increased nutrition. (b) *dsx*RNAi greatly reduces nutrition-responsive horn development in males (center) but induces it in females (right; modified after [16•]).

since divergence from a common ancestor ~5MYA has evolved perhaps the most divergent phenotypes in the entire genus: males have lost the ancestral development of (i) paired posterior head horns, as well as (ii) nutritional male horn polyphenism but have (iii) gained the development of small head horns in a novel (anterior) location on the head, whereas (iv) females have gained the

Figure 4

Control-injected (left) and *dsx*RNAi phenotypes (right) in males (top) and females (bottom) of *O. sagittarius* (modified after [16•]). See text for details.

formation of a large thoracic and a single posterior head horn [28,35].

As in *O. taurus*, *O. sagittarius* expresses male- and female-specific *dsx* splice variants [16•]. The inferred splicing patterns and translated protein sequences of all isoforms are highly similar to those in *O. taurus*, thus, the reversed sexual dimorphism in horn growth in *O. sagittarius* cannot simply be attributed to a reversal of the sex-specific splicing of Dsx isoforms in developing horns. Subsequent *dsx*RNAi studies in *O. sagittarius* revealed three major effects. (i) *dsx*RNAi substantially reduced thoracic horn size in females and induced a conspicuous protrusion in males (solid triangles in Figure 4). This indicates that in *O. sagittarius* the male isoform functions to inhibit, while the female isoform functions to promote, the growth of the thoracic horn, exactly *opposite* to its role in *O. taurus*. (ii) *dsx*RNAi caused female *O. sagittarius* to develop a pair of ectopic, male-like, anterior head horns (open triangles in Figure 9). This indicates that the female isoform functions to inhibit the growth of anterior head horns in females, identical to its role in *O. taurus*, yet involving a novel head location. (iii) Lastly, *dsx*RNAi induced surprisingly large, paired or branched ectopic horns in the posterior head in male *O. sagittarius*. These ectopic horns have no obvious homolog in wildtype males, but match the most likely ancestral character state still common among many extant members of the clade. In partial contrast, wildtype females normally do possess a large single posterior head horn, which following *dsx*RNA transformed into a branched horn (orange triangles in Figure 4). This suggests that similar to the situation for

the thoracic horn, the male-specific function of *dsx* in regulating posterior head horn development is reversed in *O. sagittarius* compared to *O. taurus*, whereas in female *O. sagittarius* it is novel, facilitating a shape change rather than horn presence or absence. Taken together, these results suggests that *dsx*RNAi phenotypes observed in *O. sagittarius* reflect a complex mosaic of at least two classes of *dsx* functions: firstly, reversed *dsx* functions relative to *O. taurus* and the presumed common ancestor, as seen in the regulation of thoracic horns (both sexes) and posterior head horns (males), and finally, novel *dsx* functions, as reflected in the regulation of anterior head horn size and posterior head horn shape in females [16•].

Doublesex has been co-opted independently in different beetle lineages into the regulation of exaggerated, nutrition-sensitive male traits

Horns, as well as sex- and morph-specific development of horns, have evolved multiple times independently in different groups of beetles, including the Scarabaeinae (to which *Onthophagus* belongs) and the Dynastinae, or rhinoceros beetles [27]. Recent work by Ito *et al*. [17•] investigated the function of *dsx* in the Japanese rhinoceros beetle (*Trypoxylus dichotomus*), a species in which males, but not females, develop greatly exaggerated head and thoracic horns as a function of larval nutrition. While male *T. dichotomus* do not exhibit a polyphenism akin to *Onthophagus taurus*, horn development within *T. dichotomus* is nevertheless extremely developmentally plastic, and as in *Onthophagus*, is predominantly affected by nutrition [17•]. Using a combination of expression and RNAi approaches, Ito *et al*. [17•] observed that as in *Onthophagus*, sex-specific *T. dichotomus* Dsx isoforms promote and inhibit the formation of head horns in males and females, respectively, though thoracic horn formation was only affected by the male Dsx isoform.

Parallel findings have now also emerged from studies in a separate beetle family, the Lucanidae or stag beetles, famous for the nutrition-dependent elaboration of mandible length in males. Mandibles, unlike horns, are not an evolutionary novelty and instead represent a highly conserved trait found in all insects; but, and like horns, mandibles in male stag beetles function in male combat over breeding opportunities, and their relative size is frequently influenced by nutrition, resulting in extreme elaboration in large, high-nutrition males of many species. Recent work by Gotoh *et al*. [36•] on the stag beetle *Cyclommatus metallifer* found that similar to horn development in *Onthophagus* and *Trypoxylus*, sex-specific Dsx isoforms promote enlarged mandibles in males but inhibit them in females. In contrast to the results on *O. taurus* (see Figure 3), *dsx* expression levels were only modestly higher in the mandibles of large males compared to those found in small males, suggesting that mandible growth is not directly linked to *dsx* expression. Instead, this study suggests that *dsx* appears to convey differential,

sex-specific sensitivity to Juvenile Hormone (JH): wild-type *C. metallifer* males respond to exogenous JH by growing larger mandibles, whereas females do not. When this experiment is repeated in *dsx*RNAi individuals the male responsiveness is muted, that of females, however, is enhanced [36•]. These results illustrate that the evolution of nutrition-dependent exaggeration of secondary sexual traits, whether recently invented or secondarily modified, has been enabled by the repeated and independent co-option of *dsx*-mediated growth regulation in diverse beetle lineages.

Future directions

Dsx is an example of a master regulatory genes, and as such is likely to possess a diverse repertoire of target genes. The body of work on onthophagine beetles reviewed here suggests that this target gene repertoire differs as a function of body region, sex, and nutrition, and, furthermore, readily diverges between species. A key goal for future research should therefore be to identify and contrast the repertoire of genes and pathways whose expression changes as a direct or indirect consequence of *dsx* up- or down-regulation in diverse male and female body regions as well as species, and ideally in both high- and low-nutrition environments.

Similarly, this research illustrates that *dsx* expression itself is influenced by nutritional conditions. However, how nutrition-sensing mechanisms (e.g. insulin signaling: [27,37]; Juvenile Hormone: [36•,38]) interact with *dsx*, and how these interactions are modulated to generate body region- and species-specific responses to nutritional variation, is completely unclear and represents an exciting new frontier for future research.

A third, intriguing new research avenue concerns the production and coordination of sex-and morph-specific *behavior* with morphology. In horn-polyphenic beetles, morphology (e.g. horned or hornless) and behavior (e.g. fighting or sneaking) must be coordinated to yield a well-integrated adaptive phenotype. Yet very different tissues and developmental processes contribute to the final product, and do so at different temporal and spatial scales. Studies are needed to explore whether genes such as *dsx* may be coordinating the development of morph- and sex-specific behavior with that of morphology.

Lastly, earlier studies have identified body size thresholds that separate horned and hornless male morphologies as a common target of evolutionary processes [39,40]. Many species and populations appear to diverge readily in this component of horn polyphenism, with the perhaps most impressive case being that of contemporary divergence in exotic *O. taurus* populations in Australia and North America, which in less than 40 years have diverged heritably in size thresholds to a degree normally observed only between species [41]. Past work has explored the role JH

signaling in threshold evolution, and found that threshold-divergent populations also diverged heritably in degree and timing of sensitivity to JH in ways that matched predictions from simple endocrine regulation models [14]. The current findings on *dsx* open up an alternative, or additional developmental mechanism, that could enable rapid divergences in body size thresholds across populations and species.

Acknowledgements

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