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Serotonin signaling suppresses the nutrition-responsive induction of an alternate male morph in horn polyphenic beetles

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Abstract

Environment-responsive development contributes significantly to the phenotypic variation visible to selection and as such possesses the potential to shape evolutionary trajectories. However, evaluation of the contributions of developmental plasticity to evolutionary diversification necessitates an understanding of the developmental mechanisms underpinning plastic trait expression. We investigated the role of serotonin signaling in the regulation and evolution of horn polyphenism in the beetle genus *Onthophagus*. Specifically, we assessed the role of serotonin in *development* by determining whether manipulating serotonin biosynthesis during the larval stage alters body size, developmental rate, and the formation of relative adult trait size in traits characterized by minimal (genitalia), moderate (elytra), and pronounced (horns) nutrition-responsive development in *O. taurus*. Second, we assessed serotonin's role in *evolution* by replicating a subset of our approaches across four species reflecting ancestral as well as derived conditions. Lastly, we employed immunohistochemical approaches to begin assessing whether serotonin may be acting via the endocrine or nervous system. Our results show that pharmacological manipulation of serotonin signaling affects overall size, developmental rate, and the body size threshold separating alternate male morphs. Threshold body sizes were affected across species, regardless of the severity of horn polyphenism, and independent of the precise morphological location of horns. However, histological assessments suggest it is unlikely serotonin functions as a neurotransmitter and instead may rely on other mechanisms that remain to be identified. We discuss the most important implications of our results for our understanding of the evolution of and through plasticity in horned beetles and beyond.

KEYWORDS
developmental plasticity, horned beetles, *Onthophagus*, threshold

1 | INTRODUCTION

Developmental or phenotypic plasticity describes the ability of individual organisms to respond to their environment by adjusting aspects of their phenotype (Pfennig et al., 2010). Such plastic responses may be adaptive or not, range from subtle responses of single traits to complex changes involving suites of phenotypes, and may or may not be reversible during an organism's lifetime (Schlichting and Pigliucci, 1998). Polyphenism, in turn, refers to a particular type of plasticity in which individuals have the potential to

develop into two or more discretely different forms, morphs, or castes (Nijhout, 2003). Adaptive developmental plasticity in general and polyphenisms in particular have attracted the attention of evolutionary developmental biologists because their study promises a deeper understanding of how developmental systems and ecological contexts interact, and how these interactions may evolve, to enable the same genotype to produce multiple adaptive forms (Sultan, 2015).

The developmental mechanisms underlying insect polyphenisms have received significant attention, with a concentration of efforts focused on the roles of juvenile hormone (JH) and ecdysteroids (ECD; Nijhout, 1994; Zera & Brisson, 2015). However, while the significance of JH, ECD, and their interplay in the regulation of insect molting and metamorphosis is without question, evidence supporting their role in the regulation of insect polyphenisms has been mixed, raising the possibility that additional endocrine and possibly non-endocrine mechanisms may exist that regulate polyphenic development in insects (Shelby, Madewell, & Moczek, 2007; Zera & Brisson, 2015). In support, recent work has identified signaling via insulin/insulin-like peptide hormones (Casasa and Moczek, 2018; Fawcett et al., 2018; Libbrecht et al., 2013), the expression of sex-specific isoforms of the somatic sex-determination gene *doublesex* (*dsx*; Kijimoto, Moczek, & Andrews, 2012; Klein et al., 2016; Ledon-Rettig, Zattara, & Moczek, 2017), and the *hedgehog* signaling pathway (Kijimoto & Moczek, 2016) as additional mechanisms underpinning polyphenism regulation. Lastly, recent work on diverse insects has begun to implicate biogenic amines such as serotonin, more commonly studied for their role in the regulation of behavior, as playing a critical role in the induction of alternate morphs. For example, topical application of serotonin, as well as the injection of serotonin precursors (5-hydroxytryptophan [5-HTP]) or serotonin receptor agonists, promoted phase transition from the solitarious to the gregarious morph in the desert locust *Schistocerca gregaria* (Anstey, Rogers, Ott, Burrows, & Simpson, 2009), while differential expression of genes associated with serotonin receptors and transporters, as well as nearly significant differences in titer, have implicated serotonin signaling in the regulation of the pea aphid wing polyphenism (Vellichirammal, Madayiputhiya, & Brisson, 2016). Here, we investigate the role of serotonin signaling in the regulation and evolution of horn polyphenism in the beetle genus *Onthophagus*.

Onthophagus are tunneling dung beetles which, with more than 2000 species worldwide, constitute one of the most speciose genera of animals (Balthasar, 1963). *Onthophagus* beetles have become attractive models in the study of developmental plasticity because males of many species respond to variation in larval nutrition by developing into one of two alternate morphs. In many species, male larvae respond to high-quality nutrition by developing into large fully horned "major" males with horns located on the head, prothorax, or both, which utilize aggressive fighting behaviors to secure mates, whereas male larvae restricted to suboptimal feeding conditions develop into smaller largely hornless "minor" males which utilize nonaggressive sneaking behaviors as adults (reviewed in Hu et al., 2020). Depending on species, the resulting horn polyphenism ranges from moderate to so severe that alternate morphs were originally described as separate species (Paulian, 1935), while in other species, the polyphenism has been secondarily lost (Casasa, Zattara, & Moczek, 2020). Furthermore, aspects of polyphenic development also diversify readily *within* species; for example, the threshold body size that separates alternate minor and major counterparts in the bull-headed dung beetle *Onthophagus taurus* has diverged rapidly among the native Mediterranean and introduced Eastern US and Western Australian populations in less than 100 generations, to a degree normally only observed among sister species (Figure S1; Moczek & Nijhout, 2003). Lastly, minor and major males also diverge in traits other than horns, such as head shape, tibial shape, testes size, ejaculate volume, or paternal care behavior, suggesting that polyphenic development in *Onthophagus* involves the nutrition-dependent adjustment of a complex syndrome of integrated morphological, physiological, and behavioral traits (reviewed in Beckers, Anderson, & Moczek, 2015). Here, we investigate the role of serotonin signaling in the regulation of relative trait size across four *Onthophagus* species characterized by varying degrees of polyphenic development.

We focused on the serotonin signaling pathway for the following reasons. First, previous work has shown that both brain and horn tissue of *O. taurus* major males exhibit increased expression of serotonin receptor 1 compared to the same tissues in hornless males (Ledón-Rettig et al., 2017), implicating serotonin signaling in the possible regulation of horn growth. Second, pharmacologically enhancing serotonin biosynthesis was found to increase most measures of aggressive behaviors in the same species, reminiscent of the effects of serotonin manipulations on phase-polyphenic locusts (Newsom, Moczek, & Schwab, 2020). Lastly, and again in the same species, a large body of past work identified key nutrition-dependent promotors and inhibitors of horn formation (Casasa & Moczek, 2018; Kijimoto and Moczek, 2016; Kijimoto et al., 2012; Ledon-Rettig et al., 2017), but a mechanistic understanding of how the threshold itself is regulated, and how this regulation might evolve, has remained largely elusive. Here, we investigated the role of serotonin signaling in the formation of multiple morphological traits exhibiting diverse degrees of nutrition responsiveness in *O. taurus*, and evaluated the effect of serotonin signaling on horn formation across three additional *Onthophagus* species characterized by moderate, extreme, and secondarily lost polyphenic development of horns. Specifically, (i) we sought to assess the role of serotonin in *development* by determining whether experimentally manipulating serotonin biosynthesis during the larval stage alters overall body size, developmental rate, and the formation of relative adult trait size in traits characterized by minimal (genitalia), moderate (elytra), and pronounced (horns) nutrition-responsive development in *O. taurus*. Second, (ii) we sought to assess the role of serotonin in *evolution* by replicating a subset of our approaches across four species reflecting ancestral (moderate polyphenism for head horns in *O. gazella*) as well as three derived conditions (pronounced polyphenism for head [*O. taurus*] and thoracic horns [*O. nigriventris*]; secondarily lost polyphenism for both horns types in *O. sagittarius*). Lastly, (iii) we employed

immunohistochemical approaches to begin assessing whether serotonin may be acting via the endocrine or nervous system. Our results show that serotonin functions to set the body size threshold separating alternate male morphs, does so across species and regardless of the severity of horn polyphenism and the precise morphological location of horns, yet is unlikely to execute this function as a neurotransmitter and instead may rely on other mechanisms that remain to be identified.

2 | MATERIALS AND METHODS

2.1 | Beetle collection and husbandry

In May 2015, adult *O. taurus* were collected from cow dung pads at Marble Hill Farm (39°3′8" N, 86°36′12" W), *O. gazella* and *O. sagittarius* were collected from Kualoa Ranch (21°31′15" N, 157°50′14" W), and *O. nigriventris* were collected from Parker Ranch (20°01′13" N, 155°40′56" W). All beetles were shipped to Bloomington, IN for rearing. Beetles were maintained simultaneously within laboratory colonies in a moist sand–soil mixture at either 24 (*O. taurus* and *O. nigriventris*) or 28°C (*O. gazella* and *O. sagittarius*) at 16 light:8 dark, and were fed cow dung ad libitum as described previously (Moczek, Hunt, Emlen, & Simmons, 2002).

To generate larvae for experimentation, adult beetles of each species were bred in plastic containers (25 cm tall × 20 cm diameter) filled 75% with moist sand–soil mixture. Three male and six female beetles were added to each container and provisioned with ~0.5 L of cow dung. Following 6 days of breeding, adult beetles were recaptured and brood balls, each containing a single larva, were collected and placed into separate plastic containers. Offspring were maintained within their natal brood balls for approximately 3 days, at which time they were transferred to individual artificial brood balls (ABBs) within 12-well tissue culture plates containing cow dung ad libitum, and maintained within stand-alone incubators at either a constant 24 (*O. taurus and O. nigriventris*) or 28°C (*O. gazella* and *O. sagittarius*). Immature *Onthophagus* complete all developmental transitions from egg to larval, pupal, and adult stages under these conditions, similar to larvae reared within their natal brood ball (Shafiei, Moczek, & Nijhout, 2001).

2.2 | Rearing and manipulation of serotonin biosynthesis

We used a pharmacological approach to experimentally manipulate serotonin biosynthesis and to evaluate its effects on growth, time to pupation, and morphogenesis in *O. taurus*. Serotonin biosynthesis requires the enzyme tryptophan hydroxylase to convert tryptophan to 5-HTP, which is then modified by the enzyme 5-HTP decarboxylase to serotonin (5-hydroxytryptamine; Vleugels, Verlinden, & Vanden Broeck, 2015). To manipulate this pathway in vivo, we haphazardly assigned larvae on the first day of the third (=final) instar to one of three treatment groups. This stage constitutes the longest phase of larval development, and it is during this stage that larvae undergo the greatest amount of growth. In one treatment group, we sought to inhibit the synthesis of serotonin from tryptophan by applying 200 μl of an aqueous 35 mM solution of α-methyl-DL-tryptophan (AMTP; #M8377; Sigma-Aldrich), a competitive antagonist of tryptophan hydroxylase, to ABB dung. By applying this solution to the dung, larvae directly ingested the AMTP through their feeding behaviors. In a second treatment group, we sought to achieve the opposite effect and enhance the synthesis of serotonin by applying 200 μl of an aqueous 100 mM solution of the serotonin biosynthetic precursor, 5-HTP (i.e., 5-hydroxy-L-trypophan; #H9772; Sigma-Aldrich) to ABB dung (as in Anstey et al., 2009; Bubak, Swallow, & Renner, 2013; Dierick & Greenspan, 2007). In a final (control) treatment group, we applied 200 μl of ddH_2O alone to each ABB. All three treatments were administered once every 3 days for the duration of the third instar. Because the addition of treatment solutions to the dung could lead to oversaturation of the ABB, thereby introducing experimental artefacts that compromise larval growth and survival, larvae were transferred to a new ABB every 6 days. Following the results of experimentation on *O. taurus*, we applied only AMTP and control treatments to larval *O. gazella*, *O. nigriventris*, and *O. sagittarius*.

2.3 | Morphometric measurements

The head horn length, elytron area, aedeagus length, and body size of all individuals were measured via a standard two-dimensional morphometric setup, including a Leica MZ16 stereomicroscope and ImageJ v. 1.44p software. We measured the left side of all symmetric structures (i.e., head horns and elytra). Head horns were measured from the outer margin of the eye to the tip of the horn, as described previously (Moczek, 2007). To obtain a measure of elytron size, we took a lateral image of each elytron, traced along its outer margins, and calculated the inner area. Aedeagus length was measured as the combined dorsal length of the paramere and phallobase, similar to Parzer and Moczek (2008). Pronotum width was used as a proxy for body size and measured as in previous studies (e.g., Emlen, 1994). All measurements were to the nearest 0.01 mm and collected by Daniel B. Schwab and Keeley D. Newsom.

2.4 | Analysis

To evaluate the effect of 5-HTP and AMTP manipulations on growth and development, we assessed the impact of these treatments on time to pupation and adult body size in male and female *O. taurus*. Because these variables failed tests for normality (i.e., adult body size) and homoscedasticity (i.e., time to pupation), they were subjected to nonparametric Kruskal–Wallis tests followed by pairwise Wilcoxon rank-sum tests to determine differences among treatment groups where necessary.

Given the sigmoidal relationship between body size and horn length for head horns of male *O. taurus* and *O. gazella*, we fit a Hill four-parameter regression model to these data:

$$\text{horn size} = \text{minimal horn size} + \frac{a(\text{body size}^b)}{c^b + (\text{body size}^b)}$$

where *a* represents the range of observed horn lengths, *b* represents the maximum slope of horn increase, and *c* represents the inflection point of the sigmoidal curve (use justified in Moczek & Nijhout, 2004). This analysis generated means and standard errors for each parameter in both species and all three treatment groups. Both AMTP and 5-HTP treatment groups were then compared against the control using Welch's *t* test. Although the thoracic horns of males in a third species, *O. nigriventris*, also demonstrate a sigmoidal relationship between body size and horn size in natural populations, in the laboratory environment, we were largely unable to rear animals to sizes falling above the body size threshold required for horn induction. Therefore, we sequentially fit different models to the body size–horn size data collected in the laboratory, and confirmed that the Hill four parameter still provided the best fit ($R^2 = 0.66$). However, our ability to resolve the full sigmoidal allometry in *O. nigriventris* rendered us incapable of analyzing the effects of serotonin manipulation on, for example, the body size–horn size threshold as in *O. taurus* and *O. gazella*. As a result, differences in the relative investment into head horns among treatment groups were examined via a residual-based analysis using a pooled sample of all AMTP and control beetles in *O. nigriventris* (as in Parzer & Moczek, 2008; Schwab & Moczek, 2014). Specifically, for all individuals, we calculated the expected horn size given body size using the parameter estimates for the horns, and then calculated the difference between this and the observed sizes to obtain residual trait values. Because the resulting values were not normally distributed, differences in residual values among treatments were assessed using a Mann–Whitney U test.

Finally, given the linear relationship between body size and both head (males and females) and thoracic (females only) horn size in *O. sagittarius*, a linear regression model was fit to all data points:

$$\text{horn size} = \text{minimal horn size} + a\ \text{body size}$$

where *a* represents the slope of the linear regression. We evaluated differences in the slope and intercept among treatments using analysis of covariance, with adult body size included as a covariate and treatments as a fixed effect. All regressions were fit using SigmaPlot v. 12.5, and tests for differences among treatment groups were conducted using SPSS statistical software v. 25.

2.5 | Sectioning, immunohistochemistry, and confocal imaging

Midprepupal *O. taurus* males predicted to metamorphose into horned males based on prepupal mass were cut between the head and thorax and fixed in PEM buffer for 1 h at room temperature (as in Gharbiah et al., 2009). Samples were dehydrated by immersion in 100% methanol overnight and rehydrated in an increasing phosphate-buffered saline (PBS):methanol series and then increasing sucrose:PBS series until reaching 40% mass/volume sucrose. Samples were kept in sucrose solution for 1–2 days, and then mounted in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek) on an aluminum slab at −20°C inside a Reichert Jung Cryocut 1800 cryostat. After at least one hour of equilibration, samples were sliced into 16–20-μm-thick sections, collected on glass slides, air-dried, and stored at −20°C until used for staining. At the time of staining, samples were rehydrated in PBS and incubated in anti-acetylated α-tubulin antibodies and 4′,6-diamidino-2-phenylindole following an established protocol (Zattara & Bely, 2015). Stained samples were imaged under a Leica SP8 laser scanning confocal microscope using LAS software.

3 | RESULTS

3.1 | Serotonin manipulations alter growth and developmental rate in *O. taurus*

We first sought to assess the effect of serotonin manipulations on aspects of growth and developmental rate. For adult body size, we found that overall growth differed significantly among treatments ($H = 13.74$, $df = 2$, $p = .001$; Figure 1a). Specifically, although there was no difference in body size between control and AMTP-treated animals ($p = 1.00$), individuals from both treatments grew to significantly larger body sizes than those treated with 5-HTP ($p = .002$ and $p = .004$, respectively). Similarly, we found that time to pupation differed significantly among treatments ($H = 52.96$, $df = 2$, $p < .001$; Figure 1b). Specifically, we found that 5-HTP-treated animals took substantially longer to reach the pupal stage than both control ($p < .001$) and AMTP ($p < .001$)-treated animals; in addition, AMTP-treated individuals developed slightly more rapidly than controls ($p = .053$).

3.2 | Inhibition of serotonin synthesis shifts the threshold for horn induction in *O. taurus*

We next addressed whether manipulating serotonin synthesis-affected aspects of morphogenesis, assessing allometric growth among three traits that are differentially responsive to nutrition, ranging from the hyperallometric growth of horns, to the isometric growth of elytra, to the hypoallometric growth of aedeagi. For horns, we found that treatment with AMTP significantly decreased the inflection point, or threshold value, of the sigmoidal allometry by a magnitude of 0.2 mm ($t = 4.63$, $df = 49$, $p < .001$), but affected no other aspect of the allometry (Figure 2a). However, we were unable to recover any effect of 5-HTP on the threshold or any other parameter. Conversely, for both the elytra and aedeagi, we recovered no effect of AMTP or 5-HTP treatment on either the intercept or slope

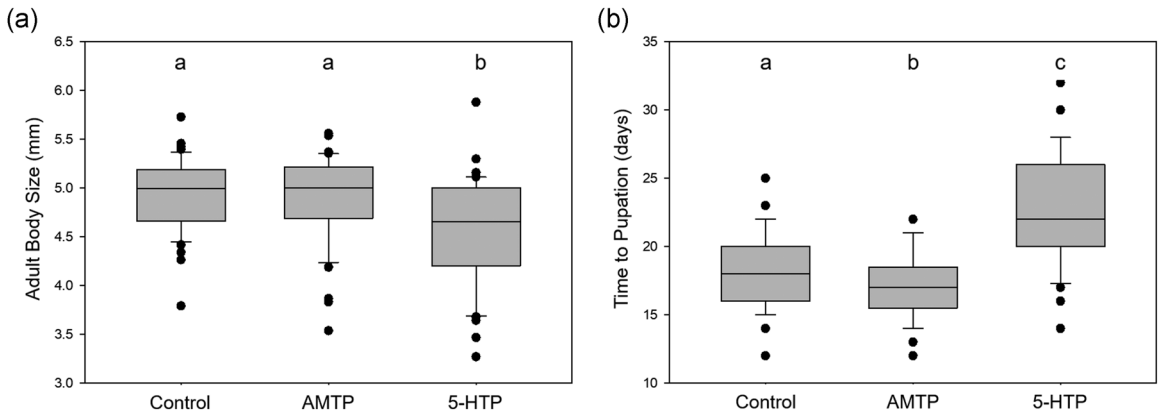

FIGURE 1 Effect of serotonin manipulations on adult body size and time to pupation in *Onthophagustaurus*. (a) *O. taurus* larvae reared under 5-HTP treatment grow to significantly smaller body sizes relative to AMTP-treated and control individuals. (b) Treatment with AMTP accelerates, and 5-HTP treatment delays, time to pupation in larvae. Letters indicate significant differences among treatment groups. 5-HTP, 5-hydroxytrypophan; AMTP, α-methyl-DL-tryptophan

of the allometries for these traits (Figure 2b,c). In combination, these results support the hypothesis that developing horn tissue is responsive to serotonin signaling, but suggests that the role of serotonin in regulating horn growth may be limited to suppressing the induction of horns in small-bodied individuals.

3.3 | Serotonin signaling affects horn growth in other polyphenic, but not in monophenic, *Onthophagus* species

We next sought to evaluate whether serotonin depletion via AMTP treatment influences horn growth in a similar manner to *O. taurus* when assessed across the diverse horn types in the *Onthophagus* phylogeny. First, we evaluated male *O. gazella*, a horn polyphenic species that is basal in the *Onthophagus* phylogeny and, similar to *O. taurus*, develops horns at the posterior of the dorsal head. We found that, as in *O. taurus*, AMTP treatment shifted the threshold at which horns are produced to a substantially lower body size ($t = 3.01$, $df = 47$, $p = .004$; Figure 3a). Next, we evaluated the effect of AMTP application on horn growth in male *O. nigriventris*, a species that possesses a single enormous and polyphenic thoracic horn. Once again, we found that AMTP significantly alters relative horn investment ($U = 551.0$, $p = .003$), promoting disproportionate horn growth in relatively small individuals that is most pronounced around the threshold at which horns develop (Figure 3b). Finally, we assessed the effect of AMTP application on head horn development in *O. sagittarius*, a species in which both males and females develop horns that scale linearly with body size, and which grow from either the head (males and females) or thorax (females only). Surprisingly, we found no effect of AMTP application on the growth of male head horns (slope: $F = 0$, $p = .993$; intercept: $F = 0.009$, $p = .925$; Figure 3c), nor on the growth of female head (slope: $F = 0.028$, $p = .868$; intercept: $F = 0.073$, $p = .789$; Figure S2a) or thoracic horns (slope: $F = 0$, $p = 1.00$; intercept: $F = 0.001$, $p = .982$; Figure S2b). Collectively, these findings suggest (i) that serotonin signaling may have been co-opted early in *Onthophagus* evolution, (ii) that serotonin function may be limited to setting the body size threshold regardless of where horns form, and (iii) that in the absence of a threshold to set, serotonin has no effect on the nutrition dependence of horn growth.

3.4 | Immunohistochemistry fails to reveal direct connection between the brain and proliferating horn tissue

We next sought to begin exploring potential mechanisms whereby serotonin may activate serotonin receptors to influence cell proliferation in horn epidermal tissue. Given that serotonin has been shown to function as both a neurohormone and a neurotransmitter (reviewed by Vleugels et al., 2015), we sought first to resolve whether the brain and proliferating horn tissue are directly apposed during prepupal development of *O. taurus*. Using cryosectioning and immunohistochemical staining, we were able to reliably visualize the growth of optic lobes from the anterior portion of the brain to the epithelium to initiate dorsal and ventral compound eye formation (Figure 4). However, despite sampling throughout the head capsules of 17 prepupal individuals, we failed to find a similar connection between any portion of the brain and proliferating horn tissue, suggesting that serotonin is unlikely to act as a neurohormone in the regulation of horn growth.

4 | DISCUSSION

Developmental plasticity, including polyphenic development, is phylogenetically ubiquitous and contributes significantly to the phenotypic variation that is visible to selection. As such, plastic phenotype expression has the potential to shape evolutionary trajectories. However, evaluation of the precise contributions of developmental

plasticity to evolutionary diversification necessitates an understanding of the developmental mechanisms underpinning plastic trait expression (Pfennig et al., 2010). Here, we sought to evaluate the role of serotonin signaling in the nutrition-dependent expression of horned beetle morphology. We find that pharmacological manipulation of serotonin signaling affects overall size and developmental rate. Furthermore, we observed that pharmacological *inhibition* of serotonin synthesis shifts the body size threshold for horn induction in three horn polyphenic species, regardless of whether horn polyphenisms are moderate or elaborate, or involve horn formation on the head or thorax. In contrast, we find that in a fourth species known to have secondarily lost polyphenic horn formation, the same experimental manipulation has no effect on horns, nor does it affect traits other than horns in *O. taurus*. Lastly, we failed to find support for the presence of serotonergic neuronal connections between the *Onthophagus* brain and horn forming epidermis, calling into question the possibility that serotonin may be acting as a neurotransmitter in this context. Below, we discuss the most important implications of our results for our understanding of the evolution of and through plasticity in horned beetles and beyond. We end by highlighting the key limitations of our current work and corresponding opportunities for future studies.

4.1 | Serotonin manipulations alter growth and developmental rate in *O.taurus*

In *O. taurus*, experimental supplementation with the serotonin biosynthetic precursor, 5-HTP (intended to *increase* serotonin biosynthesis), lengthened development time, and reduced body size, while treatment with the tryptophan hydroxylase antagonist, AMTP (intended to *decrease* serotonin biosynthesis), reduced development time without affecting body size (Figure 1). These results suggest that treatment with 5-HTP and AMTP affect larval growth dynamics in opposite directions, supporting the efficacy of our pharmacological treatments. At the same time, these results are partly inconsistent with previous studies evaluating the role of serotonin in growth regulation. For instance, serotonin signaling in the *Drosophila* brain appears to promote the secretion of insulin-like peptides that act as growth factors, thereby enlarging adult body size and reducing time to eclosion (Kaplan, Zimmermann, Suyama, Meyer, & Scott, 2008), with similar findings observed in *Aedes aegypti* mosquitoes (Ling & Raikhel, 2018). These incongruities suggest that serotonin signaling may play a general role in the regulation of growth across insect orders, but maybe evolutionarily labile with respect to its exact function within specific taxa.

4.2 | Serotonin signaling and the development and evolution of polyphenism thresholds

Earlier work on the endocrine regulation of horn polyphenic beetles championed the role of ECD and especially JH (reviewed in Shelby et al., 2007). High-dosage topical applications of the JH analog methoprene were found to induce horn tissue proliferation in presumptive hornless *O. taurus* males, which, however, invariably died during the larval to pupal transition (Emlen & Nijhout, 1999, 2001). Furthermore, different *O. taurus* populations divergent in the exact location of the body size threshold that separates alternate male morphs (Figure S1) were found to exhibit consistent differences in the sensitivity to, and developmental timing of, methoprene

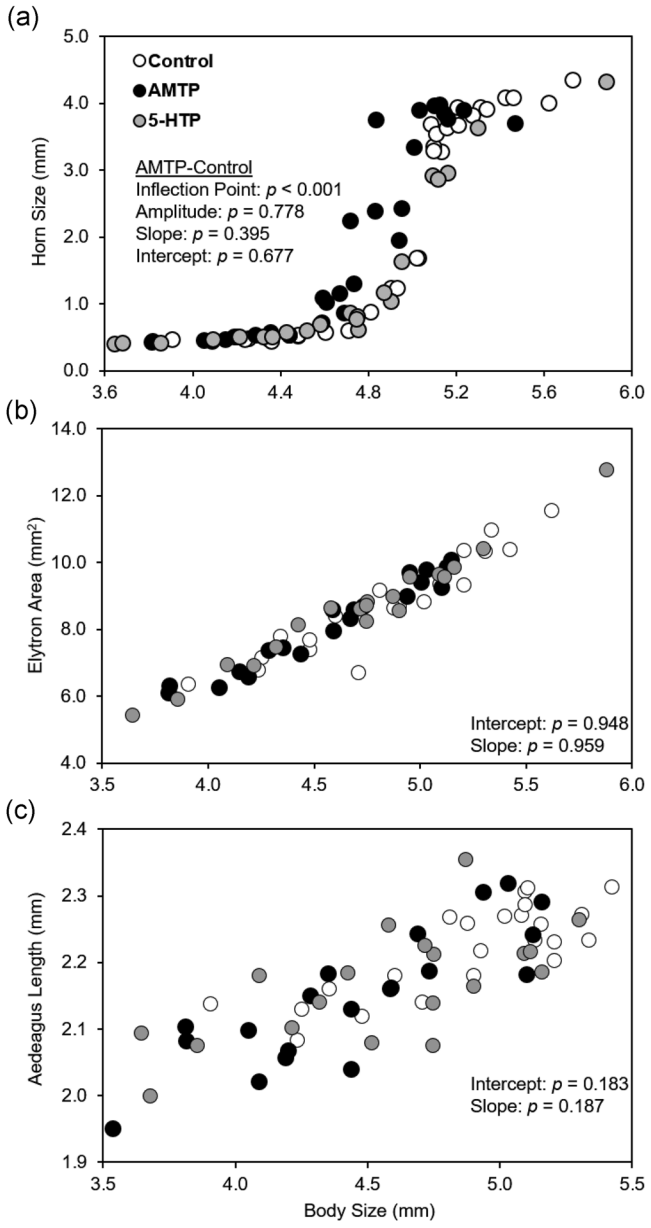

FIGURE 2 Effect of serotonin manipulations on the scaling relationship between body size (*x*-axis) and horn, elytron, or aedeagus size (*y*-axis) in *Onthophagustaurus*. (a) Treatment with AMTP significantly decreases the inflection point (i.e., threshold) of the body size–horn size allometry, but has no effect on other aspects of the allometry. In contrast, there is no effect of either AMTP or 5-HTP treatment on any aspect of the allometry for both (b) the elytron and (c) the aedeagus. 5-HTP, 5-hydroxytrypophan; AMTP, α-methyl-DL-tryptophan

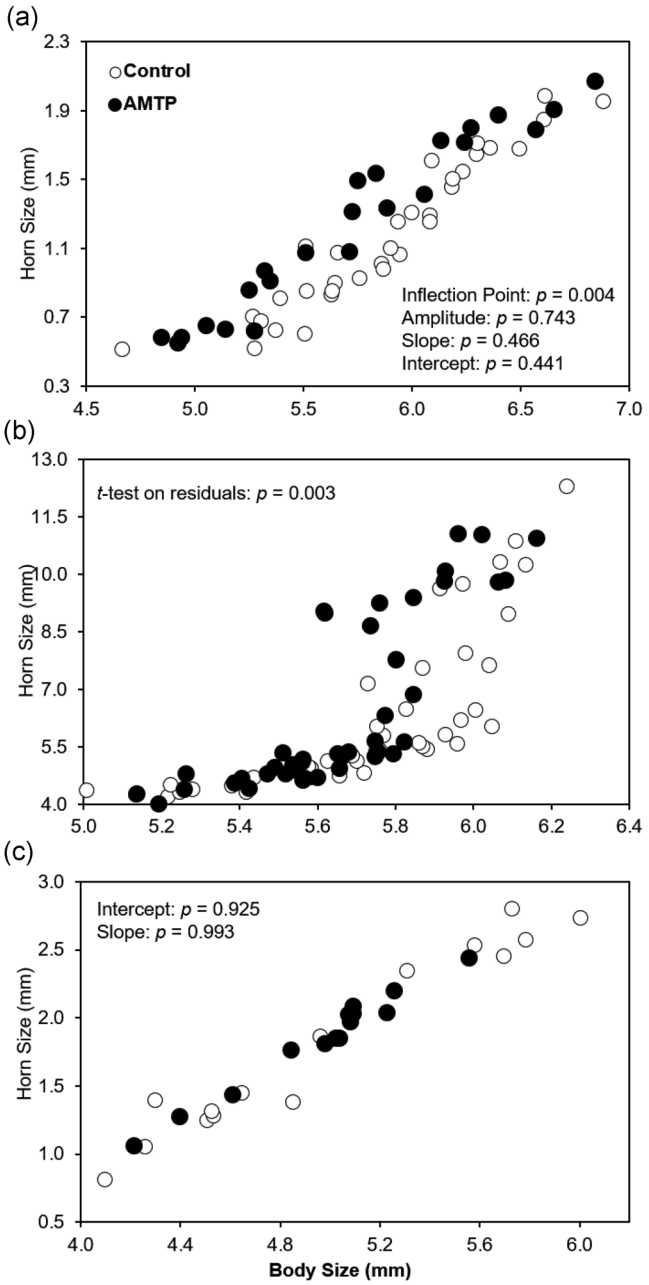

FIGURE 3 Scaling relationship between body size (*x*-axis) and horn size (*y*-axis) for male *Onthophagusgazella*, *O. nigriventris*, and*O. sagittarius* treated with AMTP. (a) Treatment with AMTP significantly decreases the inflection point (i.e., threshold), but no other aspect of the body size–horn size allometry in *O. gazella*, and (b) increases horn residual values around the threshold of *O. nigriventris*. In contrast, (c) there is no effect of AMTP treatment on any aspect of the allometry for *O. sagittarius*. AMTP, α-methyl-DL-tryptophan

applications (Moczek & Nijhout, 2002). Yet again, the vast majority of treated animals never survived high-dosage treatment, raising the possibility that the observed phenotypes may simply reflect artefactual nontarget outcomes (Zera, 2007). Since then, however, no further experimental work has been conducted on the role of JH in the regulation of polyphenic thresholds, and JH titer information, which could provide direct, in vivo support for the methoprene studies, remains lacking. This absence of critical information notwithstanding, subsequent work has increasingly treated the role of JH in the regulation of male horn polyphenisms as established fact (e.g., Hartfelder & Emlen, 2005).

More recent work has turned toward other regulatory mechanisms, combining expression and targeted functional manipulations into an increasingly complex, but also an increasingly robust model for the regulation of nutrition responsiveness and allometric scaling in horn polyphenic beetles (reviewed in Casasa & Moczek, 2019). Most importantly, a series of studies have now established the somatic sex-determination gene *dsx* as a major *promotor* of horn growth above a critical body size, the *hedgehog* signaling pathway as a key *inhibitor* of horn growth in males below the critical body size, and the insulin signaling pathway as contributing both functions simultaneously depending on male body size. What has been missing, however, are additional mechanisms that determine the precise body size threshold. The work presented here suggests that serotonin signaling may constitute such a mechanism and that this mechanism is shared across *Onthophagus* species regardless of the severity of polyphenism or the body region charged with producing horns. By extension, our results also raise the possibility that microevolutionary changes in serotonin signaling may underpin microevolutionary changes in allometric scaling and threshold divergences among allopatric populations (Figure S1).

4.3 | Serotonin signaling and the coregulation and coevolution of morphology and behavior

Insect polyphenisms involve the integrated expression of alternate morphologies, physiologies, and behaviors. In horned beetles, major males not only develop disproportionately long horns, but also exclusively engage in fighting behavior (Moczek & Emlen, 2000), while at least in *O. taurus*, also engage in a disproportionate amount of paternal care (Moczek, 1999). In contrast, minor males develop rudimentary horns, engage in sneaking behaviors, and do not assist females in brood care. These morphologies and behaviors are the product of very different developmental and physiological processes: male horns are the products of epidermal outbuddings that are initiated and grown during late larval development, and their length is fully determined in early pupae, whereas adult behavior is most likely influenced by morph-specific differentiation of function in the adult brain, which does not come into existence and does not form connections with the peripheral nervous system until mid- to late-pupal development at the earliest. This disparity in the ontogenetic origins of morph-specific morphology and behavior seems to necessitate the existence of mechanisms able to integrate morph-specific development across developmental space and time, yet the identity and nature of such mechanisms have remained elusive until recently. Studies by Kijimoto et al. (2012) and Beckers, Kijimoto, and Moczek (2017) raised the possibility that one mechanism capable of coordinating the expression of both male morphology and male behavior in horned beetles is *dsx*: not only does the expression of the male *dsx* isoform promote the formation of horn growth, it also

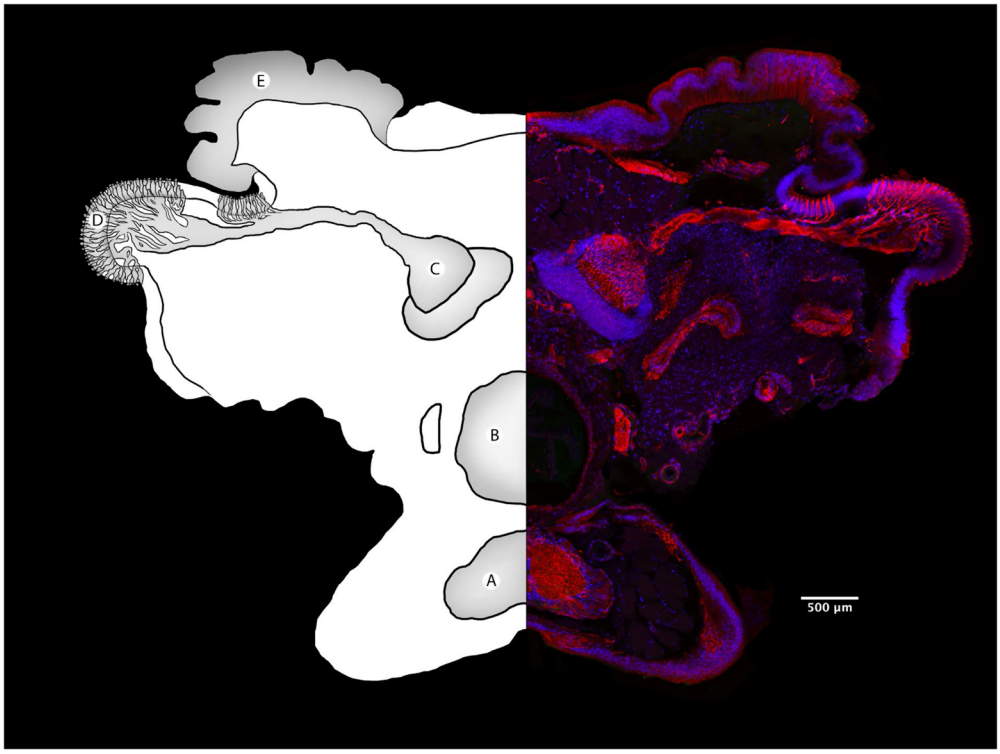

FIGURE 4 Transverse histological section through a male *Onthophagustaurus* prepupal head. Visualized is the histological structure of a large-horned male prepupa head (right) and schematic (left). The subesophageal ganglion (A) and esophagus (B) are located ventrally, with the dorsal optic lobes (C) extending outward from the anterior brain to form a pair of ventral and dorsal compound eyes (D). No such connections between proliferating horn tissue (e) and brain are observed at any point throughout the prepupal head capsule. Nucleic acids (4′,6-diamidino-2-phenylindole) are shown in blue and acetylated tubulin in red [Color figure can be viewed at wileyonlinelibrary.com]

consistently affects the expression of aggressive behavior in a sex- and morph-specific manner. Work presented here now raises the possibility that serotonin signaling may serve a similar integrating function: Newsom et al. (2020) showed that manipulating serotonin biosynthesis in adults mediates consistent changes in male aggression as a function of morph and, most intriguingly, population, while work presented here shows that similar manipulations during *larval* development alter components of adult male morphology. This raises the possibility that serotonin signaling may serve as a nexus in the coordination of morph-specific differentiation of male morphology and behavioral repertoire and suggests a candidate mechanism for the concerted evolution of phenotypic syndromes in horn-polyphenic beetles.

4.4 | Current limitations and future directions

The work presented here demonstrates a possible role of the serotonin signaling pathway in the regulation of male morphology in horned beetles, in general, and, in particular, in the specification of the body size threshold separating alternate male morphs in horn polyphenic species. However, several important limitations remain. For example, we presently lack any information on serotonin titers, or those of its precursors, in wild-type or experimentally manipulated animals, nor do we possess any insights into if and how serotonin signaling may be interacting with any of the other pathways already implicated in the regulation of horned beetle development. Most importantly, perhaps, we need to learn more about how serotonin levels physically interact with proliferating horn tissue, even though the absence of a direct neuronal connection between horn and brain tissue documented here tends to suggest that serotonin signaling may influence horn development via endocrine pathways and not as a direct result of neuronal innervation. Future work is clearly needed to address these and related questions, and to better understand the role of serotonin signaling in the development, integration, and evolution of beetle polyphenisms.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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REFERENCES

Anstey, M. L., Rogers, S. M., Ott, S. R., Burrows, M., & Simpson, S. J. (2009). Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts. *Science*, *323*, 627–630. https://doi.org/10.1126/science.1165939

Balthasar, V. (1963). *Monographie der Scarabaeidae und Aphodiidae der palaearktischen und orientalischen region (Coleoptera: Lamellicornia), Band 2, Coprinae*. Prague,Czech Republic: Verlag der tschechoslowakischen Akademie der Wissenschaften.

Beckers, O. M., Anderson, W., & Moczek, A. P. (2015). A combination of developmental plasticity, parental effects, and genetic differentiation mediates divergences in life history traits between dung beetle populations. *Evolution & Development*, *17*, 148–159. https://doi.org/10.1111/ede.12117

Beckers, O., Kijimoto, T., & Moczek, A. P. (2017). *doublesex* alters aggressiveness as a function of social context and sex in the polyphenic beetle *Onthophagus taurus*. *Animal Behavior*, *132*, 261–269. https://doi.org/10.1016/j.anbehav.2017.08.011

Bubak, A. N., Swallow, J. G., & Renner, K. J. (2013). Whole brain monoamine detection and manipulation in a stalk-eyed fly. *Journal of Neuroscience Methods*, *219*, 124–130. https://doi.org/10.1016/j.jneumeth.2013.07.006

Casasa, S., & Moczek, A. P. (2018). Insulin signaling's role in mediating tissue-specific nutritional plasticity and robustness in the horn-polyphenic beetle*Onthophagus taurus*. *Proceedings of the Royal Society of London, Series B*, *285*. https://doi.org/10.1098/rspb.2018.1631

Casasa, S., & Moczek, A. P. (2019). Evolution of, and via, developmental plasticity: Insights through the study of scaling relationships. *Integrative and Comparative Biology*, *59*, 1346–1355. https://doi.org/10.1093/icb/icz086

Casasa, S., Zattara, E. E., & Moczek, A. P. (2020). Nutrition-responsive gene expression and the developmental evolution of insect polyphenism. *Nature Ecology & Evolution*, *4*, 970–978. https://doi.org/10.1038/s41559-020-1202-x

Dierick, H. A., & Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nature Genetics*, *39*, 678–682. https://doi.org/10.1038/ng2029

Emlen, D. J. (1994). Environmental control of horn length dimorphism in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society of London, Series B*, *256*, 131–136.

Emlen, D. J., & Nijhout, H. F. (1999). Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Journal of Insect Physiology*, *45*, 45–53. https://doi.org/10.1016/S0022-1910(98)00096-1

Emlen, D. J., & Nijhout, H. F. (2001). Hormonal control of male horn length dimorphism in *Onthophagus taurus* (Coleoptera: Scarabaeidae): A second critical period of sensitivity to juvenile hormone. *Journal of Insect Physiology*, *47*, 1045–1054. https://doi.org/10.1016/S0022-1910(01)00084-1

Fawcett, M. M., Parks, M. C., Tibbetts, A. E., Swart, J. S., Richards, E. M., Vanegas, J. C., … Angelini, D. R. (2018). Manipulation of insulin signaling phenocopies evolution of a host-associated polyphenism. *Nature Communications*, *9*, 1699. https://doi.org/10.1038/s41467-018-04102-1

Gharbiah, M., Cooley, J., Leise, E. M., Nakamoto, A., Rabinowitz, J. S., Lambert, J. D., & Nagy, L. M. (2009). Fixation of *Ilyanassa* snail embryos and larvae. *Cold Spring Harbor Protocols*, *2009*(4), 4. https://doi.org/10.1101/pdb.prot5186

Hartfelder, K., & Emlen, D. J. (2005). Endocrine control of insect polyphenism. In L. I. Gilbert, K. Iatrou, & S. S. Gill (Eds.), *Comprehensive molecular insect science* (pp. 651–703). Oxford, UK: Elsevier Ltd. https://doi.org/10.1016/B0-44-451924-6/00045-4

Hu, Y., Linz, D. M., Parker, E. S., Schwab, D. B., Casasa, S., Macagno, A. L., & Moczek, A. P. (2020). Developmental bias in horned dung beetles and its contributions to innovation, adaptation, and resilience. *Evolution & Development*, *22*, 165–180. https://doi.org/10.1111/ede.12310

Kaplan, D. D., Zimmermann, G., Suyama, K., Meyer, T., & Scott, M. P. (2008). A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size. *Genes & Development*, *22*, 1877–1893. https://doi.org/10.1101/gad.1670508

Kijimoto, T., & Moczek, A. P. (2016). *Hedgehog* signaling enables nutrition-responsive inhibition of an alternative morph in a polyphenic beetle. *Proceedings of the National Academy of Sciences of the United States of America*, *113*, 5982–5987. https://doi.org/10.1073/pnas.1601505113

Kijimoto, T., Moczek, A. P., & Andrews, J. (2012). Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 20526–20531. https://doi.org/10.1073/pnas.1118589109

Klein, A., Schultner, E., Lowak, H., Schrader, L., Heinze, J., Holman, L., & Oettler, J. (2016). Evolution of social insect polyphenism facilitated by the sex differentiation cascade. *PLoS Genetics*, *12*, e1005952. https://doi.org/10.1371/journal.pgen.1005952

Ledón-Rettig, C. C., Zattara, E. E., & Moczek, A. P. (2017). Asymmetric interactions between *doublesex* and tissue-and sex-specific target genes mediate sexual dimorphism in beetles. *Nature Communications*, *8*, 14593. https://doi.org/10.1038/ncomms14593

Libbrecht, R., Corona, M., Wende, F., Azevedo, D. O., Serrão, J. E., & Keller, L. (2013). Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 11050–11055. https://doi.org/10.1073/pnas.1221781110

Ling, L., & Raikhel, A. S. (2018). Serotonin signaling regulates insulin-like peptides for growth, reproduction, and metabolism in the disease vector *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, *115*, E9822–E9831. https://doi.org/10.1073/pnas.1808243115

Moczek, A. P. (1999). Facultative paternal investment in the polyphenic beetle *Onthophagus taurus*: The role of male morphology and social context. *Behavioral Ecology*, *10*, 641–647. https://doi.org/10.1093/beheco/10.6.641

Moczek, A. P. (2007). Pupal remodeling and the evolution and development of alternative male morphologies in horned beetles. *BMC Evolutionary Biology*, *7*, 151. https://doi.org/10.1186/1471-2148-7-151

Moczek, A. P., & Emlen, D. J. (2000). Male horn dimorphism in the scarab beetle *Onthophagus taurus*: Do alternative reproductive tactics favor alternative phenotypes? *Animal Behaviour*, *59*, 459–466. https://doi.org/10.1006/anbe.1999.1342

Moczek, A. P., Hunt, J., Emlen, D. J., & Simmons, L. W. (2002). Threshold evolution in exotic populations of a polyphenic beetle. *Evolutionary Ecology Research*, *4*, 587–601.

Moczek, A. P., & Nijhout, H. F. (2002). Developmental mechanisms of threshold evolution in a polyphenic beetle. *Evolution & Development*, *4*, 252–264. https://doi.org/10.1046/j.1525-142X.2002.02014.x

Moczek, A. P., & Nijhout, H. F. (2003). Rapid evolution of a polyphenic threshold. *Evolution & Development*, *5*, 259–268. https://doi.org/10.1046/j.1525-142X.2003.03033.x

Moczek, A. P., & Nijhout, H. F. (2004). Trade-offs during the development of primary and secondary sexual traits in a horned beetle. *The American Naturalist*, *163*, 184–191. https://doi.org/10.1086/381741

Newsom, K. D., Moczek, A. P., & Schwab, D. B. (2020). Serotonin differentially affects morph-specific behavior in divergent populations of a horned beetle. *Behavioral Ecology*, *31*, 352–360. https://doi.org/10.1093/beheco/arz192

Nijhout, H. F. (1994). *Insect hormones*. Princeton, NJ: Princeton University Press.

Nijhout, H. F. (2003). Development and evolution of adaptive polyphenisms. *Evolution & Development*, *5*, 9–18. https://doi.org/10.1046/j.1525-142X.2003.03003.x

Parzer, H. F., & Moczek, A. P. (2008). Rapid antagonistic coevolution between primary and secondary sexual characters in horned beetles. *Evolution*, *62*, 2423–2428. https://doi.org/10.1111/j.1558-5646.2008.00448.x

Paulian, R. (1935). Le polymorphisme des males de cole´opteres. In G. Tessier (Ed.), *Expose´s de biome´trie et statistique biologique IV. Actualite´s scientifiques et industrielles 255* (pp. 1–33). Paris, France: Hermann.

Pfennig, D., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution*, *25*, 459–467. https://doi.org/10.1016/j.tree.2010.05.006

Schlichting, C. D., & Pigliucci, M. (1998). *Phenotypic evolution: A reaction norm perspective*. Sunderland, MA: Sinauer Associates.

Schwab, D. B., & Moczek, A. P. (2014). Resource allocation during ontogeny is influenced by genetic, developmental and ecological factors in the horned beetle, *Onthophagus taurus*. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1793). https://doi.org/10.1098/rspb.2014.1625

Shafiei, M., Moczek, A. P., & Nijhout, H. F. (2001). Food availability controls onset of metamorphosis in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Physiological Entomology*, *26*, 173–180. https://doi.org/10.1046/j.1365-3032.2001.00231.x

Shelby, J. A., Madewell, R., & Moczek, A. P. (2007). Juvenile hormone mediates sexual dimorphism in horned beetles. *Journal of Experimental Zoology B, Molecular and Developmental Evolution*, *308B*, 417–427. https://doi.org/10.1002/jez.b.21165

Sultan, S. (2015). *Organism and environment: Ecological development, niche construction, and adaptation*. Oxford, UK: Oxford University Press. https://doi.org/10.1093/acprof:oso/9780199587070.001.0001

Vellichirammal, N. N., Madayiputhiya, N., & Brisson, J. A. (2016). The genomewide transcriptional response underlying the pea aphid wing polyphenism. *Molecular Ecology*, *25*, 4146–4160. https://doi.org/10.1111/mec.13749

Vleugels, R., Verlinden, H., & Vanden Broeck, J. (2015). Serotonin, serotonin receptors and their actions in insects. *Neurotransmitter*, *2*, 1–14.

Zattara, E. E., & Bely, A. E. (2015). Fine taxonomic sampling of nervous systems within Naididae (Annelida: Clitellata) reveals evolutionary lability and revised homologies of annelid neural components. *Frontiers in Zoology*, *12*, 8. https://doi.org/10.1186/s12983-015-0100-6

Zera, A. J. (2007). Endocrine analysis in evolutionary-developmental studies of insect polymorphism: hormone manipulation versus direct measurement of hormonal regulators. *Evolution & Development*, *9*, 499–513. https://doi.org/10.1111/j.1525-142X.2007.00181.x

Zera, A. J., & Brisson, J. A. (2015). *Induction and function of polyphenic morphs: Proximate regulatory mechanisms and evolutionary implications. Integrative organismal biology* (pp. 71–90). Hoboken, NJ: John Wiley & Sons.

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