



Original Article

Serotonin differentially affects morph-specific behavior in divergent populations of a horned beetle

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Associations between animal weapons and corresponding aggressive behaviors are among the most characteristic features of species, yet at the same time their co-expression is itself often strongly dependent on context, such as male condition or population ecology. Yet the mechanisms that modulate associations between aggression, morphology, and biological context remain poorly understood. The biogenic amine serotonin has been shown to regulate a wide range of aggressive and morph-specific behaviors in diverse insect species. However, the extent to which serotonin may coordinate the expression of behavior with morphology across biological contexts remains unclear. In this study, we pharmacologically increased serotonin biosynthesis in males of the polyphenic beetle, *Onthophagus taurus*, and assessed how this manipulation affects both aggressive and non-aggressive behaviors in alternative fighter and sneaker morphs, as well as in males derived from two rapidly diverging populations characterized by disparate levels of competition for mates. We find (i) that enhancing serotonin biosynthesis increases most measures of aggressive behaviors, but influences only a subset of nonaggressive behaviors, (ii) that similar serotonin-mediated behavioral changes manifest in both morphs within populations more often than just a single morph, and (iii) that males derived from the two focal populations have diverged in their behavioral responsiveness to serotonin up-regulation. Collectively, our study suggests that serotonin signaling plays a critical role in the regulation of male behavior and its evolution, including in the context of rapid, short-term population divergence.

Key words: 5-HT, aggression, behavioral evolution, developmental plasticity, *Onthophagus* beetles, phenotypic integration

INTRODUCTION

Secondary sexual characters such as weapons (e.g., horns, antlers, and tusks) function in association with specific behavioral repertoires. Aggressive behaviors in particular play a critical role in enhancing the display and effective use of animal weapons during competitive encounters. The resulting association between exaggerated morphological traits and corresponding aggressive behaviors is commonly among the most characteristic features of particular species, yet at the same time their co-expression is itself often highly context-dependent. For instance, the expression of aggression may vary as a function of male morph (e.g., in side-blotched lizards, Sinervo and Lively 1996), population (e.g., in freshwater sticklebacks, Herczeg et al. 2009), and environmental conditions such as population density (e.g., in pseudoscorpions, Zeh 1987). Identifying the mechanisms that regulate aggression, as well as how these mechanisms may modulate associations between behavioral repertoires and morphology as a function of biological context, is

therefore critical for understanding constraints and biases in the evolution of behavior.

Many of the primary pathways that are known to modulate aggression in vertebrate taxa (e.g., catecholamines: Sallinen et al. 1998; serotonin: Karl et al. 2004; Popova 2006) have also been experimentally evaluated in insects. These pathways include brain-derived peptides, such as neuropeptide F (Dierick and Greenspan 2007), as well as various biogenic amines including the catecholamine octopamine, which has been shown to enhance aggression in red wood ants (Yakovlev 2018), *Gryllus* crickets (Rillich et al. 2011; Rillich and Stevenson 2011), and *Drosophila* (Hoyer et al. 2008). Similarly, increasing evidence suggests a particularly important role for serotonin in regulating and modulating insect aggression. For example, elevating levels of serotonin via pharmaceutical administration of its precursor, 5-hydroxytryptophan [5-HTP; as demonstrated in Dierick and Greenspan (2007) and Bubak et al. (2013)], increases expression of aggressive behaviors in flies (see also Bubak et al. 2014). Interestingly, serotonin has also been shown to play a role in the condition-dependent expression of behavior. In the phase-polyphenic desert locust, *Schistocerca gregaria*, topical application of serotonin, as well as the injection of 5-HTP

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or serotonin receptor agonists, are all sufficient to promote behavioral gregarization in otherwise solitary individuals (Anstey et al. 2009). In addition, a number of studies of social insects have begun to implicate serotonin in regulating both subcaste- and age-related divisions of labor in ants (*Pheidole dentata*: Giraldo et al. 2013; *Formica polyctena*: Wnuk et al. 2011), and bees (*Apis mellifera*: Taylor et al. 1992). Although these studies thus provide growing evidence for the role of serotonin in regulating morph- and caste-specific behavior, its role in the evolution of behavioral repertoires within and among populations remains largely unexplored.

The horn polyphenic dung beetle, *Onthophagus taurus*, is a promising study organism for understanding how serotonin may regulate aggressive behavior as a function of both morph and population. Larvae of this species develop inside subterranean masses of dung (“brood balls”) that are maternally provisioned from overlying dung pads. This allotment constitutes the only food source available to larvae throughout development, and depending on its quality and quantity, males will develop into one of two discrete, alternative morphs (Moczek 1998) with equally discrete behavioral repertoires (Moczek and Emlen 2000). Male larvae subject to high nutritional conditions develop into a large fighter morph with exaggerated bull-like horns, which guard females inside breeding tunnels and engage in aggressive interactions with other males in direct head-to-head combat. In contrast, male larvae subject to low nutritional conditions develop into a smaller sneaker morph, which only possess horn rudiments. Such hornless males adopt alternative tactics to acquire mates, including satellite behaviors and the use of intercept tunnels to circumvent large, guarding males (Moczek and Emlen 2000).

In the 1960s, *O. taurus* was introduced deliberately from its native Mediterranean range (Balthasar 1963) to Western Australia (WA) as a biocontrol measure designed to buffer the polluting effects of cattle dung (Tyndale-Biscoe 1996) as well as unintentionally to the Eastern United States (EUS; Fincher and Woodruff 1975). Since introduction, both populations have diverged rapidly and heritably in diverse traits, including horn investment, maternal behavior, hormone physiology, gene expression, and life-history traits (Moczek et al. 2002; Moczek and Nijhout 2003; Beckers et al. 2015; Macagno et al. 2016). These divergences have been attributed to substantial differences in range-specific dung beetle densities, which bring about major differences in the intensities of mate and resource competition (Moczek 2003). For example, densities of individual *O. taurus* in dung pads in WA often reach into the hundreds and low thousands, whereas corresponding EUS populations rarely exceed a few individuals. As a consequence, male–male competition for females is extreme in WA populations, but modest in EUS populations (Moczek 2003).

Previous work has shown that components of the serotonin biosynthesis pathway are differentially expressed between morphs of *O. taurus*. Specifically, both brain and horn tissue of horned males exhibit increased expression of serotonin receptor 1 compared with the same tissues in hornless males (Ledón-Rettig et al. 2017). In this study, we aimed to build on these preliminary findings and evaluate the role of serotonin in both the regulation and evolution of male behavior in *O. taurus*. Specifically, we sought to determine whether experimentally increasing serotonin biosynthesis in adult males would yield differential behavioral responses as a function of morph (i.e., horned vs. hornless) as well as population (EUS vs. WA). To do so, we assessed (i) whether pharmacologically increasing serotonin biosynthesis via 5-HTP application alters aggressive and nonaggressive components of adult male behavior (as

in Dierick and Greenspan 2007; Bubak et al. 2013, 2014), and (ii) if these behavioral alterations are biased toward or restricted to a specific morph and/or population. Specifically, we predicted that experimental alterations to serotonin biosynthesis would be most evident among aggressive male behaviors. In addition, because horned males rely exclusively on fighting for potential mates, we predicted that they would exhibit more aggressive behavior than their hornless counterparts, as well as be more responsive to experimental manipulation of serotonin biosynthesis. Similarly, because WA males are subject to higher population densities and thus intensity of competition (Moczek 2003), we predicted that WA males would exhibit greater aggression and corresponding treatment effects than EUS males. Alternatively, we reasoned that the elevated intensity of competition experienced by WA males may have resulted in the evolution of high baseline titers of endogenous serotonin in this population. If so, we predicted that WA males would exhibit increased aggression in our assays but lessened responses to experimental manipulation of serotonin titer.

MATERIALS AND METHODS

Beetle husbandry

Approximately 400 adult *Onthophagus taurus* were collected from cow dung pads near Busselton, Western Australia (−33° 39′ 8″ S, 115° 20′ 43″ E) in January 2016 and Chapel Hill, North Carolina (35° 54′ 47″ S, −79° 3′ 21″ E) in May 2016 and shipped to Bloomington, Indiana (39° 3′ 8″ N, 86° 36′ 12″ W) for rearing. Beetles from each location were maintained in separate colonies in the laboratory at 24° ± 1°C, a light:dark cycle of 16:8 h, and fed homogenized organic cow dung ad libitum following an established protocol (Moczek 2005). All dung was collected from Marble Hill Farm in Bloomington, IN. Prior to experimentation, a reserve (F₁) population was bred and maintained separately from all field-collected (F₀) individuals for both populations, and both F₀ and F₁ generations were used to generate experimental animals for this study.

To obtain offspring for experimentation, three male and six female adult beetles were added to plastic containers (26 cm tall x 20 cm diameter) filled ~75% with a moist sand-soil mixture and ~0.5 L cow dung on a weekly basis. Beetles were allowed to reproduce for 1 week, after which time brood (i.e., dung) balls containing eggs and larvae were collected. Importantly, it is unlikely that these offspring were sired by a single male within each breeding container, as the specified sex ratio limits males’ ability to monopolize multiple females simultaneously (Moczek and Emlen 2000). Larvae were transferred to 12-well plates containing dung and monitored until eclosion to adulthood (Shafiei et al. 2001).

Manipulation of serotonin biosynthesis

Following adult eclosion, males were transferred to individual 30-mL plastic cups filled halfway with soil as well as ad libitum cow dung and allowed to feed and mature in isolation for 4 days prior to experimental manipulations. Throughout this feeding period, all beetles were kept in an incubator at 25°C constant temperature, ~40% humidity, and a light:dark cycle of 16:8 h. Adult males were randomly assigned to one of two treatment groups. In one treatment group, males were fed 1 g of cow dung treated with 500 µL of a 100 mM solution of the serotonin biosynthetic precursor 5-hydroxy-L-tryptophan (5-HTP; #H9772, Sigma, St. Louis, MO) dissolved in ddH₂O. In the other (control) treatment group, males were given 1 g of cow dung treated with 500-µL ddH₂O alone. One gram of

freshly treated or untreated dung was provided once daily for three consecutive days. Animals had access to their allotment of dung until the next feeding time, ~24 h later. Due to interindividual differences in consumptive behaviors, the amount of days (i.e., 1, 2, or 3) each animal consumed their allotment of dung was recorded and included in statistical analyses (see Statistical analysis below). For experimental animals, this allotment was consumed for 2.55 ± 0.66 days on average. Animals that did not consume any treated or untreated dung throughout all 3 days were excluded from behavioral assays. Concentration and timing of application employed in our treatment matched periods of major changes in serotonin titer observed in other experimental studies of diverse insect taxa (Dierick and Greenspan 2007; Bubak et al. 2013, 2014).

Behavioral assays and analysis

Between 18 and 24 h after the third gram of dung was provided, we examined the behaviors of 5-HTP-treated ($N = 17$ for Eastern United States and $N = 20$ for Western Australia) and control ($N = 19$ for Eastern United States and $N = 19$ for Western Australia) males in interactions with wild-type (WT), size-matched male opponents of the same population from laboratory colonies. Because previous studies have shown that differences in body size between interacting males can influence the nature and extent of aggressive behavior displayed (Moczek and Emlen 1999, 2000), experimental animals were size-matched with a WT male using thorax width as a measure of body size (Emlen 1994). Animals were considered size-matched if their body size difference was less than 0.1 mm, corresponding to approximately 2% of mean thorax width. All measurements were conducted using a Leica MZ6 dissecting microscope (Leica, Heerbrugg, Switzerland), a Scion digital camera (Scion Corp., Frederick, MD), and ImageJ v1.44p software.

Behavioral assays were conducted following Beckers et al. (2017). In brief, behavioral trials were conducted in a dark room illuminated by one dim red light (25 W, General Electric brand, Party Light 25) at $25 \pm 1^\circ\text{C}$ and $32 \pm 1.5\%$ humidity. Behavioral arenas were constructed from modified plastic test tubes, allowing us to visualize all behavioral interactions (Figure 1). Following a 1-h acclimatization period within the tube, gates were removed and interactions were recorded using a tripod-mounted light-sensitive video camera (Sony, DCR-SR300) for 30 min.

We used event-logging Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba 2016) to analyze all behavioral trials. We categorized behaviors into one of two functional categories: (i) aggressive behaviors were defined operationally as all behaviors or interactions involved in the initiation or escalation of physical combat and included:

1. *Head-butts*: A head-butt was defined as a forceful, rapid, upward movement of the head toward an opponent while opponents engage in head-to-head contact. We recorded the total number of head-butts delivered to an opponent throughout the trial and calculated the percentage of fights instigated by dividing the number of first head-butts by the total fights (see below).
2. *Fights*: Fights began with a head-butt and ended when an individual retreated (see below). In addition to counting the total number of fights, we calculated cumulative fight time and the average fight duration.

In contrast, (ii) nonaggressive behaviors were operationally defined as all other behaviors observed outside direct physical combat, including:

1. *Approaches*: We counted the number of times an individual approached its opponent and initiated physical contact.
2. *Interactions*: An interaction was defined as the time period beginning when one individual initiated physical contact with its opponent and ended when an individual moved at least half a body length away from its opponent, independent of the behavior displayed. In addition to counting the total number of interactions, we calculated cumulative interaction time and the average interaction duration.
3. *Retreats*: We counted the number of times an individual moved at least half a body length away from its opponent.

One important shortcoming of our categorization is that the sets of behaviors classified as nonaggressive may still function in aggressive contexts yet only lack the physical combat component we use to identify behaviors we categorize as aggressive. We highlight the implications of this shortcoming when we juxtapose both categories for the remainder of the manuscript.

Lastly, individuals who displayed nonaggressive behaviors but did not display any aggressive behaviors scored a zero for “fights” and “head-butts” and were included in behavioral analyses, whereas individuals who did not display any behaviors during the 30-min trial were excluded from further analyses.

Statistical analysis

We analyzed the effect of 5-HTP treatment, morph, population, and their interactions, on the above 10 behavioral variables. Specifically, we used linear mixed-effect models to analyze all five continuous variables (e.g., total interaction duration, fights instigated, and average fight duration) and generalized linear mixed-effect models with negative binomial distributions to analyze count data (total approaches, total interactions, etc.). In all analyses, treatment, morph, and population were modeled as fixed effects. The number of days an experimental animal consumed their gram of

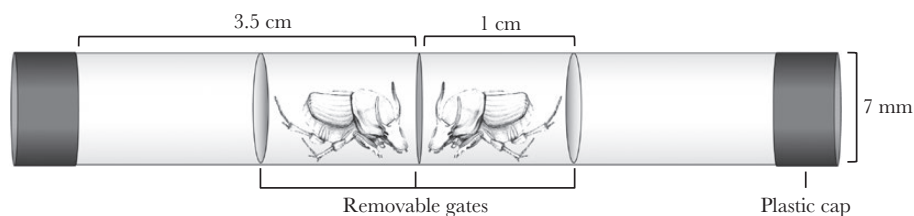


Figure 1

Schematic of behavioral arena set-up. Following acclimatization, gates were removed to allow beetles to interact throughout the full length (7 cm) of the tube. Drawing of beetles (not to scale) by Shane Richards.

Table 1

Statistical results from reduced models comparing effects of treatment, morph, population, and their interactions on nonaggressive behavior. Treatment (Trt), population (Pop), and morph were treated as fixed effects in linear mixed-effect models (cumulative interaction time, average interaction duration) and generalized linear mixed-effect models (approaches, total interactions, retreats)

Response variable	Fixed effect	Estimate	SE	df (model, error)	F ratio	<i>p</i>	Random effect	Estimate	SE
Approaches							Trt ingested	0.14	0.16
	Trt	-0.65	0.16	1, 67	16.99	<0.001			
	Morph	-0.62	0.16	1	15.93	<0.001			
Interactions									
Total interactions							Trt ingested	0.05	0.06
	Trt*Pop*Morph	0.99	0.47	1, 63	4.22	0.038			
Cumulative interaction time							Trt ingested	37806	53295
	Trt	-80.94	87.34	1, 67	0.86	0.357			
	Morph	107.81	87	1	1.54	0.21			
	Pop	-32.24	86.99	1	0.14	0.712			
Average interaction duration							Trt ingested	0.1	0.16
	Trt*Morph	-0.78	0.35	1, 64	5.04	0.028			
Retreats							Generation	0.007	0.035
	Trt*Morph	0.98	0.31	1, 64	9.74	0.003	Trt ingested	0.12	0.14
	Trt*Pop	-0.65	0.31	1	4.32	0.042			

Indicated are the response variables, fixed and random effects, estimates, standard error, degrees of freedom, *F*-ratio, and *P*-value for each factor and interaction. Significant *P*-values are bolded.

5-HTP treated or untreated dung (i.e., 1, 2, or 3), as well as the generation of the population each animal was bred from (i.e., F_0 or F_1), was modeled as random effects. Animals that did not eat any dung were excluded from the behavioral assay. Beginning with models that incorporated three-way interactions between 5-HTP treatment, morph, and population, we removed nonsignificant interactions from each model in a stepwise fashion and present the reduced models in our results. Tukey–Kramer corrections for pairwise comparisons were executed within models but not across (Indiana Statistical Consulting Center, personal communication; see Beckers et al. 2015, 2017 for additional justification) and can be found in Supplementary Tables S3 and S4. All statistical analyses were conducted using SAS statistical software (version 9.4, SAS Institute, Cary, NC).

RESULTS

Nonaggressive behaviors: approaches, interactions, and retreats

We addressed the effect of 5-HTP treatment, population, and morph on an array of nonaggressive behaviors, including approaches, interactions, and retreats. Results are summarized in Table 1 and further detailed in Supplementary Table 1. First, considering (i) approaches, we found that hornless males of both populations approached their opponent significantly more often than did horned males ($P < 0.001$), and that 5-HTP treatment significantly decreased approaches in both horned and hornless morphs ($P = 0.001$) and did so regardless of population ($P = 0.780$; Figure 2a).

We next assessed the responses of beetles for three measures of interactivity: (ii) number of interactions, (iii) average interaction duration, and (iv) cumulative interaction time. We found that (ii) number of interactions decreased only in 5-HTP treated hornless EUS males, but not their horned counterparts, nor either morph or treatment from Western Australia (WA; $P = 0.038$; Figure 2c),

resulting in a significant population \times morph \times treatment interaction. For (3) average interaction duration, we found that 5-HTP treated hornless males of either population interacted significantly longer than did hornless control males, resulting in a significant morph \times treatment interaction ($P = 0.028$; Figure 2d). In contrast, (4) cumulative interaction time was unaffected by morph, treatment, or population ($P = 0.220$, $P = 0.358$, and $P = 0.712$, respectively).

Additionally, we observed a morph \times treatment interaction for (5) retreats: hornless 5-HTP treated males of either population, but not horned males, exhibited a reduction in the frequency of retreats ($P = 0.003$, Figure 2b). Finally, we found that among control individuals, males derived from high competition WA populations retreated significantly less often than their corresponding EUS counterparts, whereas 5-HTP treatment reversed this ranking, causing 5-HTP treated EUS males to retreat less frequently than 5-HTP treated WA males (treatment \times population, $P = 0.042$, Figure 2b).

Aggressive behaviors: fights and head-butts

We next addressed the effect of 5-HTP treatment, morph, and population on the (vi) total number of fights, (vii) average fight duration, (viii) cumulative fight time, (ix) total head-butts, and (x) fights instigated. Results are summarized in Table 2 and detailed in Supplementary Table 2.

First, we found that the (vi) number of fights increased in all 5-HTP treated animals ($P < 0.001$; Figure 3a) regardless of morph and population. In contrast, for both (vii) average fight duration ($P = 0.025$, Figure 3c) and (viii) cumulative fight time ($P = 0.029$, Supplementary Figure 1), we recovered a significant population \times treatment interaction: assessed for either variable, 5-HTP treated EUS individuals fought longer than control males, whereas WA males exhibited no such treatment effect.

The number of (ix) total head-butts and (x) fights instigated was also affected. Both horned and hornless males of either population

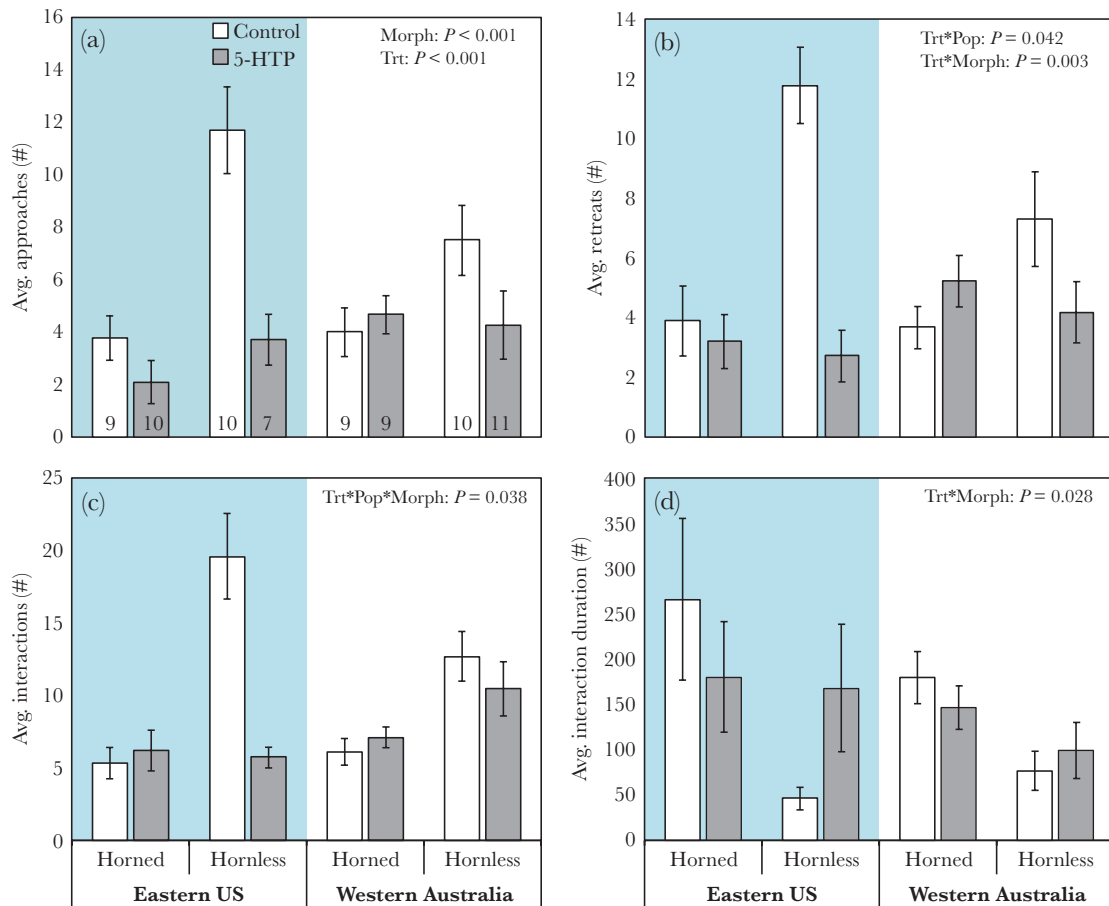


Figure 2

Nonaggressive behaviors of experimental animals as a function of treatment, population, and morph. (a) Number of approaches. Hornless males approach their opponent more than horned males, whereas treatment with 5-HTP decreases approaches. (b) Number of retreats. Hornless 5-HTP treated males retreat less than control counterparts. In addition, Eastern US (EUS) individuals retreat more than Western Australia (WA) individuals under control conditions, whereas under 5-HTP treatment, the directionality of this result is reversed. (c) Number of interactions. Hornless EUS males treated with 5-HTP decreased their number of interactions, but this response was not observed in either morph from WA. (d) Average interaction duration. Regardless of population, 5-HTP treatment increased the average interaction time in hornless males but decreased this time in horned males. Responses of control and 5-HTP treated individuals are shown in white and gray boxes, respectively. EUS population is distinguished from WA by background shading. Numbers at the bottom of each bar in (a) represent the sample sizes for that bar and all corresponding bars in (b)–(d). Error bars represent standard error. Significance of treatment (Trt), population (Pop), and morph, and any significant interactions, are indicated with P values.

who were treated with 5-HTP threw significantly more head-butts during fights ($P < 0.001$; Figure 3b) and were more likely to instigate fights ($P < 0.001$; Figure 3d). Finally, the percentage of instigated fights also exhibited a weak trend towards a population \times treatment effect: control WA males tended to instigate more fights than their EUS counterparts, but 5-HTP treatment increased fight instigation more so in EUS than WA ($P = 0.086$; Figure 3d).

DISCUSSION

In this study, we sought to investigate the role of serotonin signaling in the regulation of male–male interactions and aggressive behavior in the horned beetle, *O. taurus*. To do so, we assessed whether pharmacological application of the serotonin precursor, 5-HTP, alters the behavioral repertoires specific to horned or hornless male morphs, or both, and if these alterations may be biased as a function of population. We predicted that (i) experimental alterations to serotonin biosynthesis would be most evident among aggressive

male behaviors, (ii) the behavior of horned males would be disproportionately affected by our manipulations compared with hornless males, and (iii) WA males would exhibit higher baseline aggression than EUS males, and respond to pharmacological manipulation of serotonin signaling in an altered fashion compared to their EUS counterparts. Our results provide partial support for these predictions.

5-HTP application alters both aggressive and nonaggressive behaviors

With the exception of cumulative interaction time, all behaviors assessed in this study were affected by our manipulation of serotonin biosynthesis in at least one population or morph. Specifically, 5-HTP application resulted in a consistent, significant increase in four of six aggressive behaviors (i.e., first head-butts, total head-butts, total fights, and fights instigated) regardless of morph or population. In contrast, our pharmacological manipulations consistently altered the expression of only one nonaggressive behavior

Table 2

Statistical results from reduced models comparing effects of treatment, morph, population, and their interactions on aggressive behavior. Treatment (Trt), population (Pop), and morph were treated as fixed effects in linear mixed-effect models (cumulative fight time, average fight duration, fights instigated) and generalized linear mixed-effect models (total head-butts, total fights)

Response variable	Fixed effect	Estimate	SE	df (model, error)	F ratio	<i>p</i>	Random effect	Estimate	SE
Head-butts									
Total head-butts							Trt ingested	<0.001	<0.001
							Generation	<0.01	0.043
First head-butt	Trt	2.61	0.38	1, 67	46.12	<0.001	—	—	—
	Trt	2.01	0.32	1, 67	39.17	<0.001	—	—	—
	Morph	0.42	0.22	1	3.77	<i>0.057</i>	—	—	—
Fights									
Total fights	Trt	0.88	0.23	1, 67	14.5	<0.001	—	—	—
Fights instigated	Trt	0.46	0.06	1, 64	66.19	<0.001	—	—	—
	Trt*Pop	0.2	0.11	1	3.04	<i>0.086</i>	—	—	—
Cumulative fight time	Trt*Pop	2.24	1	1, 64	4.97	0.029	—	—	—
Average fight duration	Trt*Pop	1.94	0.85	1, 64	5.26	0.025	—	—	—

Indicated are the response variables, fixed and random effects, estimates, standard error, degrees of freedom, *F*-ratio, and *P*-value for each factor and interaction. Significant *P*-values are bolded. Marginally significant *P*-values are italicized.

(i.e., decreased approaches), but differentially affected all other nonaggressive behaviors as a function of morph, population, or both.

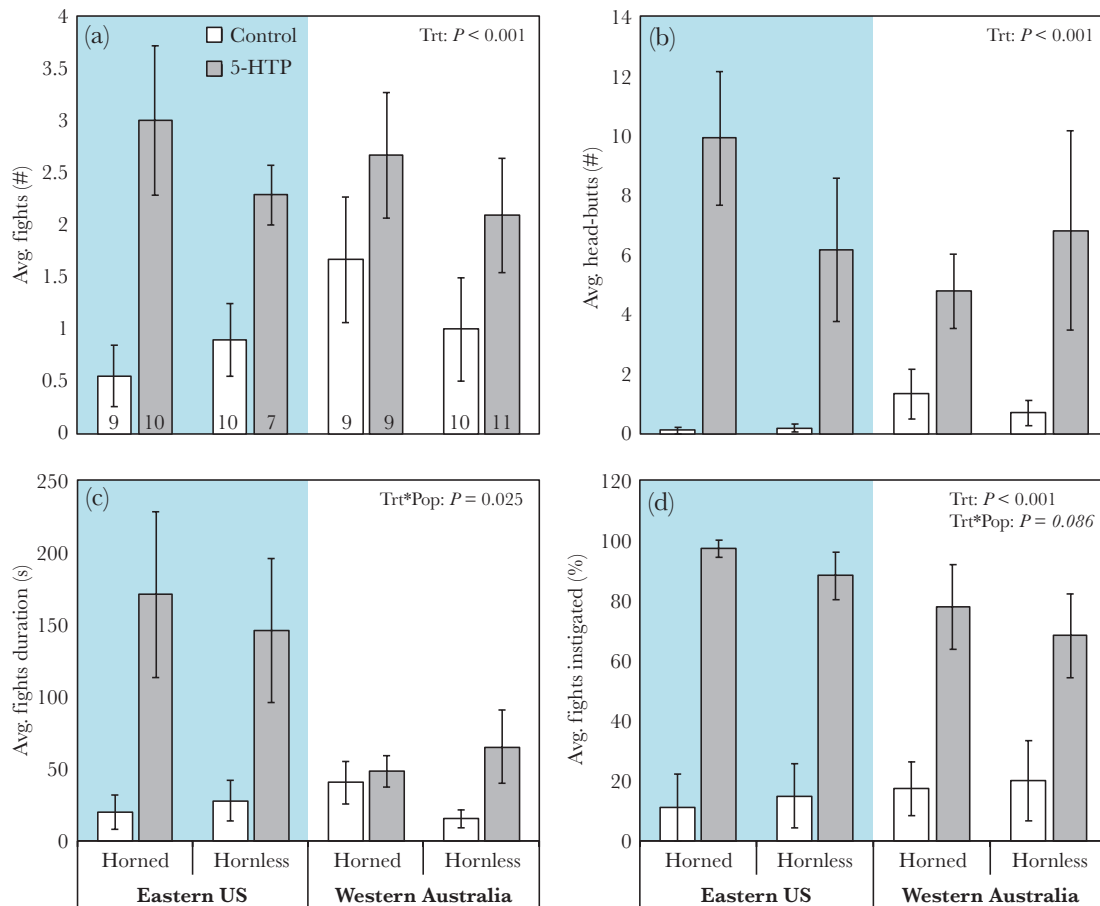
Although we presently lack direct measurements of serotonin titers under natural and experimental conditions, the results of our 5-HTP manipulations are consistent with, and expand upon, a growing body of evidence linking serotonin signaling to aggression, which in insects has thus far been limited largely to studies of flies and crickets (e.g., Dierick and Greenspan 2007; Dyakonova and Krushinsky 2013). For instance, recent studies in the fruit fly, *Drosophila melanogaster*, demonstrate that activating serotonin biosynthesis in the brain via pharmacological (i.e., 5-HTP application) and genetic methods increases both the number of encounters as well as the escalation to and number of fights among males (Dierick and Greenspan 2007; Alekseyenko et al. 2010, 2014). These findings have been largely replicated in stalk-eyed flies, *Teleopsis dalmani*, where treatment with 5-HTP similarly increases aggression and the likelihood of winning fights while decreasing retreats in size-matched bouts between males (Bubak et al. 2014). In male crickets (*Gryllus bimaculatus*), activation of serotonin biosynthesis via injection with 5-HTP has been shown to increase some aspects of aggression (e.g., fight duration), while decreasing (i.e., number of attacks) or having no effect on (i.e., likelihood of winning), the frequency of others (Dyakonova and Krushinsky 2013).

At the same time, our results support the hypothesis that serotonin signaling plays an important role in the context-dependent regulation of nonaggressive behaviors. That serotonin influences a wide array of nonaggressive behaviors, such as sleep (Yuan et al. 2006), hunger (Albin et al. 2015), and courtship (Becnel et al. 2011) is now well-established. However, the role of serotonin in regulating the nonaggressive behaviors addressed here (approaches, interactions, and retreats) has received mixed support among insect taxa. For instance, in addition to suppressing the likelihood of retreat or escape responses, serotonin signaling appears to enhance the general activity level of crickets (Dyakonova and Krushinsky 2013; but see Stevenson et al. 2000). However, experimentally

reducing neurotransmission in the serotonergic cells of *Drosophila* has no effect on either levels of activity or the number of aggressive encounters among male flies (Alekseyenko et al. 2010). Collectively, these results further emphasize the diverse regulatory potential of serotonin signaling across taxa, yet also underscore the continued need to evaluate this potential on a case by case basis.

5-HTP application influences aggressive behaviors irrespective of morph

Perhaps the most striking feature of *O. taurus* development is its polyphenic nature, with males developing into either a horned morph that relies exclusively on aggressive behaviors to access mates, or a hornless morph that relies more heavily on nonaggressive sneaking behaviors (Moczek and Emlen 2000). We predicted that highly aggressive horned males would be disproportionately responsive to 5-HTP application compared with horned males. However, aside from a marginally significant finding that horned males threw more first head-butts regardless of population (Supplemental Figure 2), levels of aggressiveness among male morphs responded similarly to 5-HTP application. Instead, the only significant two- or three-way interactions that we recovered involved three non-aggressive behavioral traits: 5-HTP application (i) decreased average interaction duration for horned males, but increased it for hornless males, (ii) dramatically decreased retreats for small males but had little effect on horned males, and (iii) decreased the total number of interactions for hornless EUS males. In each case, 5-HTP treatment induced hornless males to exhibit nonaggressive behaviors at levels similar to those of horned control males, whereas the same treatment had little to no effect on horned males. Our findings thus indicate that although 5-HTP treatment modulates aggressive behaviors in *O. taurus* males, it does so largely irrespective of male morph. Whether this is because these morphs share similar concentrations of endogenous serotonin, or because 5-HTP application was simply

**Figure 3**

Aggressive behaviors of experimental animals as a function of treatment, population, and morph. (a) Total number of fights. Treatment with 5-HTP increased total fights across both populations and morphs. (b) Total number of head-butts. Animals treated with 5-HTP threw significantly more head-butts and instigated more fights than controls. (c) Average fight duration. Eastern US (EUS) males treated with 5-HTP demonstrated increased fight duration, but this effect was not as pronounced in Western Australia (WA). (d) Fights instigated. Animals treated with 5-HTP instigated significantly more fights than controls. Responses of control and 5-HTP individuals are shown in white and gray boxes, respectively. EUS population is distinguished from WA by background shading. Numbers at the bottom of each bar in (a) represent the sample sizes for that bar and all corresponding bars in (b)–(d). Error bars represent standard error. Significance of treatment (Trt), population (Pop), and morph, and any significant interactions, are indicated with P values. Marginally significant P values are italicized.

insufficient to promote morph-specific differences, is presently unclear, but could be elucidated with direct measurements of serotonin titer. Conversely, our findings suggest that hornless males express many putatively nonaggressive behaviors, such as approaches and interactions, at higher levels than their horned counterparts. These results are consistent with recent findings by Beckers et al. (2017) and suggest that descriptors of horned males as “aggressive fighters” and hornless males as “non-aggressive sneakers” may represent an overly simplistic dichotomy. Indeed, behavioral observations show that hornless males will engage readily in prolonged fights as long as they encounter similar-sized opponents, and switch to nonaggressive sneaking behaviors only after they lose a fight, in contrast to horned males that fight exclusively and simply withdraw from further engagements when they lose (Moczek and Emlen 2000). Hornless males are thus fully capable of engaging in prolonged aggressive interactions, yet unlike their horned counterparts, do so in a more context-dependent manner. This reassessment raises the possibility that morph-specific behavioral repertoires

may emerge through the context-dependent modulation of an otherwise shared set of pathways rather than the strict morph-specific deployment of select pathways.

Response to 5-HTP application is partly dependent on source population

Since being introduced from their native Mediterranean range in the late 1960s (Balthasar 1963), exotic EUS and WA populations have diverged rapidly in a number of morphological and life history traits [reviewed in Macagno et al. (2016); see also Casasa and Moczek (2018)]. This divergence has been attributed to significant differences in local dung beetle densities, leading to highly elevated competition for access to mates and dung in WA (Moczek 2003). We therefore predicted that WA males should exhibit heightened levels of aggression and be either more or less affected by our pharmacological manipulations compared with EUS males. We recovered partial support for this hypothesis, finding that population interacted with treatment and/or morph in two nonaggressive (i.e., total interactions, retreats) and three aggressive (i.e., cumulative

fight time, average fight duration, and fights instigated) behaviors. Intriguingly, and in support of our alternate prediction, we found that WA males were less responsive to 5-HTP treatment than those from EUS. For example, we found that 5-HTP treatment increased cumulative fight time and average fight duration irrespective of morph, but this response was disproportionately stronger in males from the EUS compared with the WA population. Additionally, we found that hornless EUS males retreated far less in response to 5-HTP treatment than their WA counterparts.

Few studies have successfully linked population divergence in suites of behavioral traits with variation in particular physiological pathways. For instance, although some studies have characterized among-population divergence in behavioral repertoires (e.g., in zebrafish: Moretz et al. 2007; sticklebacks: Herczeg et al. 2009), these studies often fail to specify the underlying mechanisms. Conversely, separate sets of studies have attempted to characterize differential patterns of gene expression in populations experiencing divergent ecological conditions (e.g., *Drosophila*: Matzkin 2012; killifish: Whitehead et al. 2012), but either lack the ability to make clear linkages to particular behavioral differences, or behavioral differences are poorly characterized. Perhaps the best example of this linkage between behavior and mechanism has emerged from studies of allopatric populations of Amargosa pupfish, *Cyprinodon nevadensis*, which vary in aggression due to differential expression of the peptide hormone arginine vasotocin (Lema 2006). Although our findings demonstrate that divergent *Onthophagus* populations are differentially responsive to 5-HTP treatment, future progress in understanding the evolution of these populations may come from interrogating candidate pathways that are either already known to be involved in modulating aggression, such as octopamine (see above) and neuropeptide F (Dierick and Greenspan 2007), or have been identified as being differentially expressed among the EUS and WA populations in transcriptomic studies (Pespenti and Moczek in review).

Conclusions

Our findings add the horn-polyphenic dung beetle, *O. taurus*, to a small but growing list of insect taxa whose behavioral repertoire, and in particular behaviors related to male aggression, may be modulated through the serotonin signaling pathway. At the same time, our results extend current understanding of the possible significance of serotonin signaling by illustrating that a key component of the serotonin biosynthesis pathway, 5-HTP, may play a role in short-term behavioral divergences between exotic populations in existence for less than 50 years and approximately 100 generations. Alternatively, it is possible that serotonin may be interacting with other pathways which are then manifesting in behavioral changes. Manipulation of serotonin signaling, for example, has been shown to precipitate changes to circulating juvenile hormone (Rachinsky 1994), and can suppress the release of insulin-like peptides (Luo et al. 2012), both of which can also alter components of behavior associated with aggression (reviewed in Fahrback 1997). Lastly, this study presents many promising opportunities for further elucidating the role of serotonin signaling in *Onthophagus* behavior and evolution. For instance, although our methodology and results are fully consistent with those of previous studies in insects (Dierick and Greenspan 2007; Alekseyenko et al. 2010; Bubak et al. 2014), future studies should (i) further validate these findings by directly measuring endogenous serotonin titers among morphs and populations of *O. taurus*, and (ii) evaluating the efficacy of 5-HTP

in elevating the serotonin titer of *O. taurus*. Furthermore, given the diversity of pathways already documented to underlie morphological differentiation in *Onthophagus* (reviewed in Casasa et al. 2017), including serotonin-responsive pathways such as juvenile hormone (Rachinsky 1994; Shelby et al. 2007) and insulin signaling pathways (Snell-Rood and Moczek 2012; Casasa and Moczek 2018; reviewed in Fahrback 1997), future research is now able to explore if and how serotonin signaling interacts with these pathways, and if and how changes in these interactions enable morph-, population-, and species-specific differentiation in the co-expression and co-evolution of behavioral and morphological phenotypes.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Newsom et al. 2019.

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