Animal Behaviour 132 (2017) 261–269

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

doublesex alters aggressiveness as a function of social context and sex in the polyphenic beetle *Onthophagus taurus*

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ARTICLE INFO

Article history:
Received 21 December 2016
Initial acceptance 27 February 2017
Final acceptance 21 June 2017

MS. number: A16-01092R2

Keywords:
behavioural regulation
fighter
mate acquisition behaviour
phenotypic plasticity
polyphenism
sneaker

Despite sharing nearly the same genome, individuals within the same species can vary drastically in both morphology and behaviour as a function of developmental stage, sex or developmental plasticity. Thus, regulatory processes must exist that enable the stage-, sex- or environment-specific expression of traits and their integration during ontogeny, yet exactly how trait complexes are co-regulated and integrated is poorly understood. In this study, we explore the developmental genetic basis of the regulation and integration of environment-dependent sexual dimorphism in behaviour and morphology in the horn-polyphenic dung beetle *Onthophagus taurus* through the experimental manipulation of the transcription factor *doublesex (dsx)*. The gene *dsx* plays a profound role in the developmental regulation of morphological differences between sexes as well as alternative male morphs by inhibiting horn formation in females but enabling nutrition-responsive horn growth in males. Specifically, we investigated whether experimental downregulation of *dsx* expression affects male and female aggressive and courtship behaviours in two social contexts: interactions between individuals of the same sex and interactions between males and females. We find that *dsx* downregulation significantly alters aggressiveness in both males and females, yet does so differently for both sexes as a function of social context: dsx^{RNAi} males exhibited elevated aggression towards males but showed reduced aggression towards females, whereas dsx^{RNAi} females became more aggressive towards males, while their aggressiveness towards other females was unaffected. Moreover, we document unexpectedly high levels of female aggression independent of *dsx* treatment in both wild-type and control-injected individuals. Lastly, we found no effects of dsx^{RNAi} on courtship and mating behaviours. We discuss the role of *dsx* in the regulation of sex-specific and plastic behaviours, the unexpectedly high levels of aggression of hornless dsx^{RNAi} males in relation to the well-established description of the hornless sneaker phenotype and the potential ecological function of high female aggression.

Individuals within the same species can vary drastically in both morphology and behaviour as a function of developmental stage, sex or developmental plasticity. Such divergences often involve complex suites of traits related to morphology, physiology and behaviour, and regulatory processes must exist that enable the stage-, sex- or environment-specific expression of traits and their integration during ontogeny. Advances in sequencing technology and the experimental analyses of pathway functions have greatly contributed to the understanding of the conditional differentiation of specific traits (e.g. Kijimoto et al., 2014; Ledón-Rettig & Moczek, 2016; Ledón-Rettig, Zattara, & Moczek, 2017), yet how multiple, complex traits may be co-regulated in a context-dependent manner remains poorly understood. In particular, the developmental genetic regulation of complex behavioural phenotypes and their integration with morphology can provide important insights in how developmental processes enable genotype–phenotype maps to be sensitive to context during ontogeny, and how such context sensitivity may diversify during evolution.

Some of the most thorough understanding of the genetic regulation and integration of behaviour and morphology comes from studies on the sexual dimorphism in *Drosophila melanogaster*. Male *D. melanogaster* appear and behave distinctly different from

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http://dx.doi.org/10.1016/j.anbehav.2017.08.011
0003-3472/

females (Hall, 1994; reviewed in Billeter, Rideout, Dornan, & Goodwin, 2006). Both morphological and behavioural sex differences have been related to the sex-specific gene expression and mRNA splicing of two prominent members of the sex-determination gene cascade: the transcription factors *doublesex* (*dsx*) and *fruitless* (*fru*; reviewed in Billeter et al., 2006). Specifically, *fru* is a well-established major regulator of sex-specific behaviours in *Drosophila* (e.g. Demir & Dickson, 2005; Manoli, Meissner, & Baker, 2006), and while sex-specific expression of *dsx* isoforms was thought to regulate primarily the development of somatic sex differences (e.g. Christiansen, Keisman, Ahmad, & Baker, 2002; Cline & Meyer, 1996), more recent studies indicate additional functions in the regulation of sex-specific behaviours through sex-biased development of various parts of the nervous system (e.g. Billeter et al., 2006; Bray & Amrein, 2003; Kimura, Hachiya, Koganezawa, Tazawa, & Yamamoto, 2008; Pan & Baker, 2014; Rezával et al., 2012; Rideout, Billeter, & Goodwin, 2007).

Despite these advances, however, little is known about the degree to which *dsx* and *fru* in *Drosophila* reflect parts of a conserved developmental machinery responsible for integrating sex-biased behaviour and morphology across taxa, or alternatively, represents an idiosyncrasy unique to higher flies. In addition, due to its strength as a laboratory organism, including robustness against environmental perturbations, findings in *Drosophila* provide little insight into the mechanisms that underlie the environment-sensitive nature of sex-biased differentiation otherwise widespread in insects and other taxa (e.g. alternative male mating tactics: Danforth, 1991; Godin, 1995; Kolluru & Grether, 2005). Thus, if and how environmental modulation of sex differences in morphologies and behaviour utilizes the same or different pathways compared to taxa with more canalized sexual differentiation remains largely unknown. In this study, we explore the developmental genetic basis of the regulation and integration of environment-dependent sexual dimorphism in behaviour and morphology in the horn-polyphenic dung beetle *Onthophagus taurus*.

Based on larval feeding conditions, *O. taurus* males develop into either large adult males possessing long, paired head horns (major males) or small males with greatly reduced or rudimentary horns (minor males; Moczek, 1998; Moczek & Emlen, 1999). These alternative male phenotypes also differ distinctly in their mate acquisition behaviours (Moczek & Emlen, 2000): major males use their horns in tunnels to aggressively fight other males by head butting and shoving their opponents to gain and maintain access to breeding females. Major males fight opponents of any size until one of them is expelled from the tunnel. Once defeated, major males retreat, do not re-engage with their opponent, and attempt to leave the area (Moczek & Emlen, 2000). Minor males, in contrast, display a more versatile behavioural repertoire. Like horned major males, hornless minor males will engage in the same head-to-head combat behaviour when encountering a male opponent. In fights between same-sized minor males, these contests typically result in prolonged aggressive battles indistinguishable from those between same-sized horned major males (Moczek, 1999; Moczek & Emlen, 2000). However, in cases in which minor males encounter larger males, they invariable lose fights and are evicted from the tunnel (Moczek & Emlen, 2000). In striking contrast to major males, upon defeat, minor males do not attempt to leave the area but instead reattempt to access male-guarded females through the use of a series of alternative behaviours. These include repeated attempts to pass guarding males, the utilization of naturally occurring tunnel intersections to access females while avoiding guarding males, the active digging of horizontal intercept tunnels and the solicitation of matings from females collecting dung above ground but underneath the dung pad (Moczek & Emlen, 2000). Minor males frequently alternate among these sneaking behaviours over the course of hours following the initial defeat. Experimental data suggest that both morphs are the product of intrasexual disruptive selection: while the possession of long horns greatly improves success in direct head-to-head combat and is thus favoured in major males, 'hornlessness' greatly improves manoeuvrability and agility inside tunnels and is thus advantageous in minor males (Madewell & Moczek, 2006; Moczek & Emlen, 2000).

Female *O. taurus*, in contrast, are always hornless regardless of larval feeding conditions and adult body size. Furthermore, females do not exhibit preferences for males based on their fighting success with other males (McCullough & Simmons, 2016), male morphology in general and male horn phenotype in particular (Kotiaho, 2002), and do not engage in alternate behaviours as a function of their own body size. Thus, in contrast to their male counterparts, females appear behaviourally and morphologically monomorphic. Most importantly, the existence of phenotypically monomorphic females alongside strikingly polyphenic males necessitates the existence of mechanisms that are able to integrate morphology with behaviour during ontogeny not only as a function of sex, but also as a function of external conditions, which is the focus of this study.

The transcription factor *dsx* plays an important role in the regulation of sex- and morph-specific development of horns in *O. taurus* (Kijimoto, Moczek, & Andrews, 2012): alternative splicing of *dsx* results in one male-specific isoform and multiple female-specific isoforms of *dsx* (*dsxm*, *dsxf*), with the male isoform promoting horn formation in males, while the female isoforms inhibit horn formation in females. Furthermore, *dsxm* appears to play a critical role in regulating nutrition-sensitive formation of horns in minor and major males. *dsxm* is differentially expressed in developing horn primordia as a function of larval nutrition, and RNAi-mediated downregulation of *dsxm* drastically reduces nutrition-responsive horn growth in large males, while leaving the modest horn growth in smaller males largely unaffected. In striking contrast, downregulation of *dsxf* in female *O. taurus* induces nutrition-responsive development of male-like head horns (Kijimoto et al., 2012) in typically hornless females. Thus, *dsx* plays a critical role in the regulation of both sexual dimorphism and male polyphenism in *O. taurus*, and independent studies in rhinoceros beetles (Ito et al., 2013) and stag beetles (Gotoh et al., 2014) have since confirmed that sex-specific *dsx* isoforms have repeatedly evolved to not just inhibit the formation of weapons in females, but also to disproportionately enhance their growth in high-nutrition males.

Here, we investigate whether *dsx* also regulates behavioural phenotypes in male and female *O. taurus*, and may thus serve as a nexus coordinating and integrating the development of suites of behavioural and morphological traits as a function of sex and environment. More specifically, we investigated whether experimental downregulation of *dsx* expression affects male and female aggressive and courtship behaviours, focusing on two social contexts: interactions between individuals of the same sex and interactions between males and females. Since major horned males acquire mates exclusively by fighting their opponents, we predicted that if *dsx* coordinates and integrates aggressive behaviours with the corresponding morphology, then dsx^{RNAi} males, which exhibit greatly reduced horns, should also exhibit reduced aggressiveness. Conversely, we predicted that dsx^{RNAi} females should exhibit elevated aggressiveness, given that dsx^{RNAi} induces the expression of horns in otherwise entirely hornless females. Furthermore, because minor males mate opportunistically under a wide range of opportunities compared to major males (Moczek & Emlen, 2000), we predicted that less aggressive hornless dsx^{RNAi} males might court females more frequently, whereas horned dsx^{RNAi} females

might be more aggressive towards males, thereby reducing courtship interactions and/or would be courted less often because of their more 'male-like' phenotype.

METHODS

Animal Husbandry

Approximately 400 adult beetles were collected near Chapel Hill, North Carolina, U.S.A. in 2011 and maintained in the laboratory following Moczek and Nagy (2005) and Beckers, Anderson, and Moczek (2015). In brief, we bred field-collected animals to obtain larvae for *dsx*-knockdown and control injections (see below). We transferred larvae to 12-well plates as described in Shafiei, Moczek, and Nijhout (2001) and monitored larval development until time of injection (see below). Injected larvae were returned to 12-well plates until eclosion to adulthood. Adults were then transferred to plastic cups (946 ml) filled with a soil/sand mixture (2:1) and cow manure collected from an organic pasture near Bloomington, Indiana, U.S.A. as food source. Beetles were kept in an environmental chamber at $24 \pm 1\,^{\circ}C$ constant ambient temperature, approximately 40% humidity and a 16:8 h light:dark cycle.

Generation of dsx^{RNAi} *Animals*

We utilized the same approach as detailed in Kijimoto et al. (2012) to generate genetically manipulated (*dsx*^{RNAi}) and control-injected individuals (dsRNA derived from a vector sequence and buffer). Details on cloning, generation of *dsx*^{RNAi} and control constructs, injection procedures and knockdown validation via qPCR can be found in Kijimoto et al. (2012). Additional knockdown validation of the same injection procedure via RNAseq can be found in Ledón-Rettig et al. (2017). Key procedures are outlined in brief below.

Experimental Downregulation of dsx *via RNA Interference*

Onthophagus dsx isoforms are expressed in a strictly sex-specific manner and to varying degrees in different tissue types, although the highest expression levels are typically detectable in sexually dimorphic tissues (Kijimoto et al., 2012). We used the same dsRNA construct to downregulate *dsx* as used in Kijimoto et al. (2012) and Ledón-Rettig et al. (2017). This construct effectively and systemically downregulates all previously isolated alternative male and female *dsx* isoforms as previously validated by QRT-PCR (relative to wild type; Kijimoto et al., 2012) and RNAseq (relative to control-injected individuals; Ledón-Rettig et al., 2017). Importantly, injection of this construct into the haemolymph of larvae corresponds to a systemic knockdown of *dsx* and yields both highly consistent and penetrant morphological phenotypes in both sexes. All *dsx*^{RNAi} individuals used in the present study ($N = 78$) presented phenotypes that fully replicated those described in previous work (Kijimoto et al., 2012; Ledón-Rettig et al., 2017). To produce dsRNA, we used plasmid vectors (Agilent, Santa Clara, CA, U.S.A.) containing different *dsx* fragments (either a part shared between sexes or one specific to females) following procedures detailed in Kijimoto et al. (2014). To execute *dsx*^{RNAi} knockdowns, we then injected 0.5 μg of dsRNA into larvae during the first 5 days of the final, third instar.

Control Injections

Control animals were reared under the same conditions as *dsx* dsRNA-injected animals but were instead injected with dsRNA from a 220 bp PCR product derived from a pSC-A vector as described in Kijimoto et al. (2014). We injected 1 μg of dsRNA into larvae during the first 5 days of the final, third instar. We refer to these control-injected animals as 'controls' throughout the text.

Behavioural Assays

We examined the behaviours of *dsx*^{RNAi} and control males and females in two social contexts: (1) same-sex interactions in which *dsx*^{RNAi} males ($N = 22$; control: 17) or *dsx*^{RNAi} females ($N = 18$; control: 18) were paired with wild-type (WT) males or females, respectively; and (2) mixed-sex interactions in which *dsx*^{RNAi} males ($N = 17$; control: 16) or *dsx*^{RNAi} females ($N = 21$; control: 22) were paired with WT females or WT males, respectively. In mixed-sex and same-sex control trials, control males and females interacted with WT animals. Since the behaviours that we were interested in quantifying are expressed in interactions with another animal and not in isolation, we used this experimental design to generate a baseline level of the intensity of the behavioural interactions of interest (control trials) to which we compared the effects of *dsx* downregulation.

Adult age, defined as the period between adult emergence from the brood ball and the date of testing, was broadly overlapping across treatment groups. Specifically, in same-sex trials, adult ages of *dsx*^{RNAi} males ranged between 8 and 16 days (control: 9–23 days) and those of *dsx*^{RNAi} females ranged between 14 and 25 days (control: 16–22 days). In the mixed-sex trials, age for *dsx*^{RNAi} males ranged from 8 to 18 days (control: 10–17 days) and for *dsx*^{RNAi} females from 13 to 21 days (control: 16–21 days). Females were tested at a slightly older age than males to ensure sexual receptivity. All WT females in the mixed-sex trials had been adult for 3+ weeks and were virgins to increase the likelihood that courtship and/or mating would occur.

All males, regardless of body size and horn length, initially engage in head-to-head combat when confronted with a rival male (Moczek & Emlen, 2000). To standardize and maximize our ability to detect potential differences in male (and female) aggressiveness towards same-sex beetles as a consequence of *dsx* knockdown, we size-matched male and female beetles in the same-sex experimental (*dsx*^{RNAi} versus WT) and control trials (control versus WT). We used thorax width as a measure of body size (Emlen, 1994) and categorized competing individuals of equal size when their thorax width difference was less than 0.1 mm (corresponding to approximately $\leq$2% of mean thorax width).

In contrast, we did not size-match beetles in the mixed-sex trials because these trials were designed to test the effect of *dsx*^{RNAi} on sexual interactions and specifically courtship rather than aggression, and previous work has failed to detect any differences in courtship behaviour as a function of male and/or female size (Moczek & Emlen, 1999).

For all behavioural trials, we placed two beetles into a clear polycarbonate tube with an inner diameter of 7 mm (same-sex trials) or 9.5 mm (mixed-sex trials) and a length of 10 cm (Fig. 1). Because males stand upright on their hindlegs when they are courting and mating females, we used these slightly wider tubes for

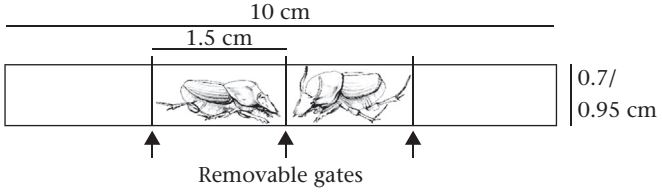

Figure 1. Schematic of experimental tunnel set-up (not drawn to scale). Note that larger diameter tunnels (0.95 cm) were used in mixed-sex trials to provide additional room for upright male mounting of the female. Drawings by Shane Richards.

mixed-sex trials. The diameters of both tubes fall within the approximate width of natural underground tunnels of this species (Moczek & Emlen, 2000). Tubes were glued to a 30 cm wooden paint-mixing stick, which allowed us to position tubes horizontally, using a customized scaffold. Tubes were capped on both ends with removable plastic caps constructed from microcentrifuge tubes (VWR International, Radnor, PA, U.S.A.) to facilitate loading and unloading of beetles into the tube. During acclimation, beetles were separated by nontransparent barriers ('gates') inserted through slits cut into the tube: one barrier was positioned to separate beetles prior to trials and one barrier was placed 1.5 cm behind each beetle to prevent them from moving towards the far end of the tube during acclimation (Fig. 1).

All behavioural experiments were conducted in a dark room illuminated by two dim red lights (25 W, Party light 25, General Electric, Boston, MA, U.S.A.) and an ambient temperature of 26 ± 2 °C. We acclimatized beetles in this room for 1 h prior to trials. We started the trials by removing all three barriers, enabling beetles to interact with each other as well as giving them the full length of the tube to move around while interacting with each other. We used a tripod-mounted light-sensitive video camera (Sony DCR-SR300, Sony Corp., Tokyo, Japan) to record behaviours of both beetles for 30 min. This period was generally sufficient to observe both aggressive and courtship interactions (O. M. Beckers, personal observation). After each trial, beetles were preserved in 95% ethanol (i.e. each beetle was tested only once). Lastly, we rinsed the tubes and gates between trials, first with liquid soap solution, then with 95% ethanol and finally with tap water to remove chemical cues left by beetles previously tested in the tube.

Behavioural Analysis

Video analyses of beetle behaviour started 1 min after removing the barriers from the tube to exclude any artefactual behaviours induced by our handling. In most trials, one individual would approach and engage its opponent until one of the two individuals retreated. Interactions consisted of fighting, involving lowering the head, advancing forward and engaging in head-butting behaviour (Moczek & Emlen, 2000), pushing, courting using elytral drumming or copulating. Interactions generally recurred multiple times during the 30 min trial. In this study, we aimed to compare levels of aggression, courtship and willingness to mate between treatment groups, focusing our analysis on the following behaviours.

(1) Number of approaches. Here, we determined which individual (*dsx*^{RNAi}, control, WT) approached its opponent and initiated physical contact. Physical contact with the opponent led to display of either aggressive or reproductive behaviours. If both individuals approached each other at the same time, the approach was counted for both individuals.
(2) Interaction duration. Beetles in physical contact with each other can engage in fighting (most of the time) and/or courting. Interaction duration represents the cumulative time that individuals spent in physical contact with each other during the trial, independent of the behaviour displayed and was defined as starting when one individual initiated physical contact with its opponent until one individual moved at least half a body length away from its opponent.
(3) Courtship. Males initiated mating with females by rapidly drumming their foreleg tarsi on the pronotum or elytra of the female. We noted the occurrence and measured the cumulative duration of this 'drumming' for each trial.
(4) Mounting. After successful courtship, males mounted females from behind while standing on their hindlegs. We counted the occurrence of mounting attempts in each trial.
(5) First head butt. Fights involved head butting the opponent. Head butts involve a quick, typically upward movement of the head towards the opponent's body, often coinciding with a forward movement. For each fighting bout, we measured which beetle head-butted its opponent first. This kind of behaviour typically escalated fights, leading to more head butting.
(6) Number of head butts. We counted the total number of head butts (including the first head butt; see above) that each individual delivered to its opponent during the 30 min trial.
(7) Retreat. We counted the number of times each individual moved at least half a body length away from its opponent during the 30 min trial. If both individuals retreated at the same time, the retreat was counted for both animals.

Ethical Note

Our research adhered to the ASAB/ABS guidelines for the use of animals in research, the legal requirements of the U.S.A., and all guidelines of Indiana University. Animals were carefully collected and handled, and maintained in the laboratory under proper conditions. We did not observe any physical injuries on any of the beetles as a result of the trials or rearing procedures. Tested animals were preserved in ethanol and stored at Indiana University as voucher specimen.

Statistical Analysis

Depending on the kind of trial, each individual contributed up to seven data points (corresponding to behaviours 1–7 outlined above). Some individuals did not display all analysed behaviours in a given trial and thus scored a zero for a subset of behaviours. For each behaviour, we first tested whether the treatment (*dsx*^{RNAi} or control) significantly affected the probability of displaying a given behaviour by comparing the ratio of displaying and not displaying (= 'zeroes') individuals between treatments using Fisher's exact tests. We found no significant difference in the number of displaying individuals for any of the analysed behaviours. We excluded behavioural data from *dsx*^{RNAi} and control individuals that scored a zero from further analysis. In addition, we excluded eight data points (out of a total of 1200+ data points collected across all trials) with extremely high values for a given behaviour (e.g. number of head butts), because these values were at least twice as high as the highest value of all the other data for this behaviour from the same treatment (*dsx*^{RNAi} or control). Depending on the trial, final sample sizes for each behaviour ranged from 10 to 22.

To quantify the effect of treatment on the different behaviours, we used generalized linear models (GLM) with Poisson distributions to compare the number of approaches, first head butts, total head butts and retreats in both same-sex and mixed-sex trials. Interaction time was analysed using ANOVA. The variance and distribution of all data sets justified the use of parametric testing procedures. For same-sex trials (male versus male, female versus female) opponents were size-matched and we included treatment, body size of the *dsx*^{RNAi} or control individual, ambient temperature, behavioural response of the WT opponent and adult age (measured as days after adult emergence from brood ball) and all two-way interactions between treatment and the other variables as fixed effects in the model. For mixed-sex trials, the animals were not size-matched and we additionally included body size of the WT opponent and its interaction with treatment as fixed effects in the

model. We removed nonsignificant interactions and corresponding single-factor variables except for treatment in a stepwise fashion from the model and present the reduced models in our results. One of our models indicated a marginally significant effect of treatment (first head butts in male–male interactions; see below). For these data, we calculated the effect size using Cohen's *d* (Cohen, 1988) to assess the validity of the significance. All statistical comparisons were performed using JMP (version 11.0, STATA Corp., College Station, TX, U.S.A.).

RESULTS

For each experiment, we present below (and summarized in Tables 1, 2), the effects of treatment (i.e. dsx^{RNAi} or control) on each type of behaviour, followed by the most relevant interaction terms that emerged during our analysis. For a complete delineation of each statistical model, including all interaction terms, see Supplementary Material (Tables S1–S20).

Same-sex Trials: Male–Male Trials

First, we examined the effect of *dsx* downregulation on male aggressive behaviour in the context of male–male interactions. Trials in which dsx^{RNAi} males fought size-matched WT males took significantly longer than those in which control males fought size-matched WT males (ANOVA: $F_{1,36} = 11.52$, $P < 0.001$; Fig. 2a). dsx^{RNAi} males initiated fights by throwing the first head butt significantly more often when interacting with WT males compared to control males interacting with WT opponents (Fig. 2a). Our analysis indicated a borderline significant treatment effect (GLM: $\chi^2_1 = 3.60$, $P = 0.0576$), however, the relatively large effect size of our data (Cohen's $d = 0.76$) provides confidence that this *P* value identified a meaningful treatment effect. Similarly, dsx^{RNAi} males directed significantly more head butts towards their WT opponent than did control males (GLM: $\chi^2_1 = 11.96$, $P < 0.001$; Fig. 2a). In contrast, dsx^{RNAi} males approached their WT male opponent significantly less often than did control males (GLM: $\chi^2_1 = 7.15$, $P = 0.008$; Fig. 2a), possibly a reflection of the longer durations that dsx^{RNAi} males stayed engaged in fights. Downregulation of *dsx* had no effect on the average number of retreats from fights (Table 1). Overall, the increased duration of fighting, the number of fights initiated and the total number of head butts indicates that dsx^{RNAi} increased the aggressiveness of males towards other males.

On rare occasions, we observed males courting other males and engage in elytral drumming, a behaviour typically displayed in the context of courtship with females. This behaviour occurred at low frequency regardless of treatment and was observed in three out of 23 trials in which a WT male drummed on a dsx^{RNAi} male and in one out of 18 trials in which a control male drummed on a WT male. These frequencies did not differ significantly (Fisher exact test: $P = 0.618$) between treatments.

Table 1
Summary statistics for treatment and significant interactions between treatment and other factors for same-sex interactions

Response variable	Fixed effect	*df* (model, error)	χ^2 or *F* ratio	*P*
Male vs male				
Interaction duration	Treatment (Trt)	1, 36	11.52	**<0.001**
Approaches	Trt	1	7.15	**0.008**
First head butt	Trt	1	3.60	**0.058**
Total head butts	Trt	1	11.96	**<0.001**
	Trt∗size dsx^{RNAi}	1	8.31	**0.004**
	Trt∗age	1	4.15	**0.042**
Retreats	Trt	1	0.04	0.852
Female vs female				
Interaction duration	Trt	1, 33	0.23	0.639
Approaches	Treatment	1	5.07	**0.024**
	Trt∗age	1	9.96	**0.002**
First head butt	Trt	1	0.02	0.90
Total head butts	Trt	1	0.07	0.799
	Trt∗size dsx^{RNAi}	1	73.39	**<0.001**
Retreats	Trt	1	0.19	0.662
	Trt∗age dsx^{RNAi}	1	6.82	**0.009**

WT: wild type. ANOVAs were used to analyse interaction time and GLMs for all other behaviours. Significant *P* values are shown in bold. For detailed statistical tables for the complete models, see Supplementary Material.

Table 2
Summary statistics for treatment and significant interactions between treatment and other factors for mixed-sex interactions

Response variable	Fixed effects	*df* (model, error)	χ^2 or *F* ratio	*P*
dsx^{RNAi} male vs female				
Interaction duration	Treatment (Trt)	1,30	0.07	0.790
Approaches	Trt	1	0.02	0.898
	Trt∗size dsx^{RNAi}	1	9.49	**0.002**
First head butt	Trt	1	0.38	0.540
	Trt∗size WT	1	8.25	**0.004**
	Trt∗age	1	9.06	**0.003**
Total head butts	Trt	1	8.39	**0.004**
	Trt∗size dsx^{RNAi}	1	9.72	**0.002**
	Trt∗size WT	1	17.64	**<0.001**
	Trt∗age	1	4.82	**0.028**
Retreats	Trt	1	9.39	**0.002**
dsx^{RNAi} female vs male				
Interaction duration	Trt	1, 39	0.10	0.759
Approaches	Trt	1	0.90	0.342
	Trt∗age	1	7.77	**0.005**
First head butt	Trt	1	4.83	**0.028**
	Trt∗size WT	1	15.29	**<0.001**
Total head butts	Trt	1	19.41	**<0.001**
	Trt∗size dsx^{RNAi}	1	12.53	**<0.001**
	Trt∗size WT	1	62.93	**<0.001**
Retreats	Trt	1	11.89	**<0.001**
	Trt∗size WT	1	8.10	**0.004**
	Trt∗age	1	12.16	**<0.001**

WT: wild type. ANOVAs were used to analyse interaction time and GLMs for all other behaviours. Significant *P* values are shown in bold. For detailed statistical tables for the complete models, see Supplementary Material.

Female–Female Trials

Interactions between females were surprisingly aggressive and aggression levels indicated by the number of first head butts and total number of head butts were comparable to those of male–male interactions (Supplementary Fig. S1). As with male–male interactions, female–female interactions occurred between size-matched individuals, but unlike male–male interactions, we did not detect any significant difference in the time that females interacted aggressively with each other, the number of first head butts, the total number of head butts or the number of retreats from fights (see Table 1) between treatment groups. However, the number of approaches towards the opposing WT female was significantly higher for dsx^{RNAi} females than for control females (GLM: $\chi^2_1 = 5.07$, $P = 0.024$; Fig. 2b). We did not observe any courtship or mounting behaviour in any of the female–female trials. Thus, even though female aggression towards same-sex opponents was similar in intensity to that measured for males, it appeared largely unaffected by the downregulation of *dsx*.

Male–Female Trials: Courtship Behaviour

In the third experiment, we analysed the effect of *dsx* downregulation on male behaviour when interacting with WT females.

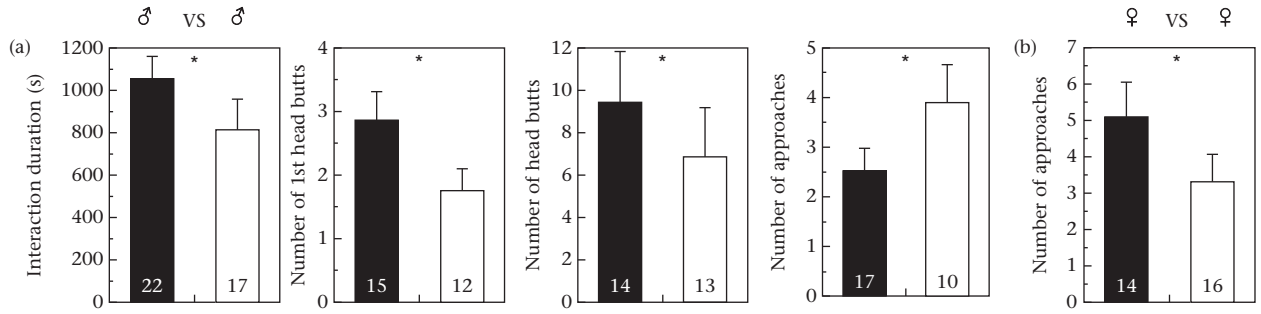

Figure 2. Significant effects of dsx^{RNAi} on behaviour in same-sex interactions. Bars indicate average behavioural responses (±SEM) of dsx^{RNAi} beetles (■) and control beetles (□) when paired with size-matched wild-type (WT) opponents of the same sex. (a) Male–male interactions; (b) female–female interactions. An asterisk indicates a significant treatment effect ($P < 0.05$) and numbers at bottom of each bar indicate sample sizes.

We did not observe any courtship or mounting in any of the trials involving dsx^{RNAi} males and WT females ($N = 17$). Similarly, we observed courtship and mounting behaviours by only one control male that was paired with a WT female ($N = 16$). These frequencies did not differ significantly between treatments (Fisher exact test: $P = 0.48$). Unexpectedly, we observed in one of the trials that a WT female drummed briefly on the pronotum of a dsx^{RNAi} male.

Next, we compared the effect of *dsx* downregulation on female behaviour when paired with WT males. The proportion of WT males courting and mounting dsx^{RNAi} and control females did not differ significantly (courtship: dsx^{RNAi}: 7 out of 21; control: 8 out of 22; Fisher exact test: $P = 1.0$; mounting: dsx^{RNAi}: 3 out of 21; control: 5 out of 22; Fisher exact test: $P = 0.698$). Similarly, the average time WT males spent courting females from each treatment group did not differ significantly (t test: $t_{13} = -0.819$, $P = 0.43$). Overall, we failed to detect any significant effects of *dsx* downregulation on courtship and mating behaviour in males or females.

Male–Female Trials: Aggressive Behaviour

Male–female interactions were surprisingly aggressive. Frequently, males attacked females, but overall females fought males more vigorously (see Supplementary Fig. S1). Male aggression towards WT females was not affected by dsx^{RNAi} with regard to interaction time, approaches towards the female or number of first head butts thrown (see Table 2 for P values). However, dsx^{RNAi} males directed significantly fewer head butts towards WT females (GLM: $\chi^2_1 = 8.39$, $P = 0.004$; Fig. 3a) and retreated significantly more often (GLM: $F_{1,2} = 9.39$, $P = 0.002$; Fig. 3a) from interactions with females than did control males. Thus, the overall effect of *dsx* downregulation in males when paired with WT females was a reduction of aggression.

In contrast, dsx^{RNAi} females were significantly more aggressive towards WT males than were control females: dsx^{RNAi} females initiated fights with males significantly more often by throwing the first head butt (GLM: $\chi^2_1 = 4.83$, $P = 0.028$; Fig. 3b) and also headbutted males more often (GLM: $\chi^2_1 = 19.41$, $P < 0.001$; Fig. 3b) than did control females. While the interaction time with males and the number of female approaches towards males did not differ significantly between dsx^{RNAi} and control females (Table 2), dsx^{RNAi} females retreated significantly more often from WT males than did control females (GLM: $\chi^2_1 = 11.89$, $P < 0.001$; Fig. 3b), consistent with a reduced interest of dsx^{RNAi} females in potential male courtship advances. Thus, when paired with the opposite sex, *dsx* downregulation had opposite effects on males and females: dsx^{RNAi} males showed reduced aggression towards females, whereas dsx^{RNAi} females became more aggressive towards males.

Significant Interactions

Aside from the treatment effects outlined above, we also detected several significant interactions between treatment and specific fixed effects in our analysis. The most prominent interactions occurred between treatment and size and between treatment and age of either the dsx^{RNAi} animal or its opponent (summarized in Tables 1 and 2; see additional statistical tables in the Supplementary Material for other interactions not discussed here).

Interactions Between dsx^{RNAi} *Treatment and Body Size*

In mixed-sex interactions, regardless of the sex of the dsx^{RNAi} individual, and in contrast to control individuals, dsx^{RNAi} individuals (1) were significantly less likely to escalate fights with

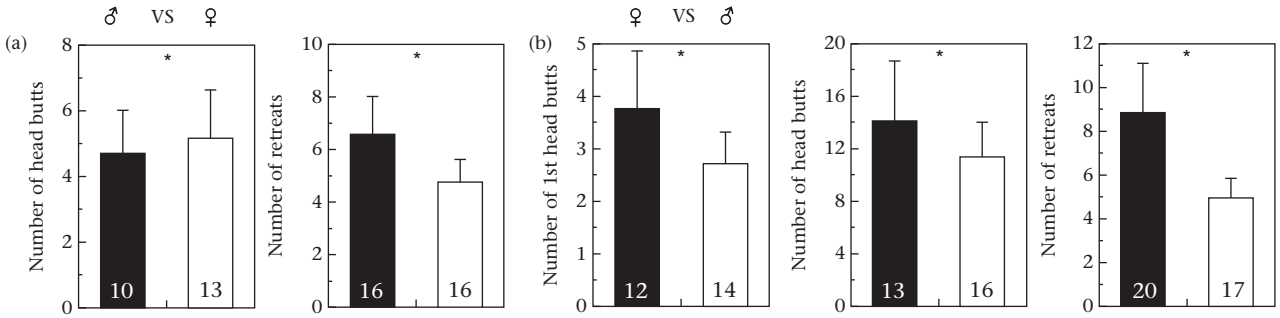

Figure 3. Significant effects of dsx^{RNAi} on behaviour in mixed-sex interactions. Bars indicate average responses (±SEM) of dsx^{RNAi} beetles (■) and control beetles (□) when paired with wild-type (WT) beetles of the opposite sex. (a) dsx^{RNAi} males paired with WT females. (b) dsx^{RNAi} females paired with WT males. An asterisk indicates a significant treatment effect ($P < 0.05$) and numbers at bottom of each bar indicate sample sizes.

larger opponents (GLMs: $P \leq 0.004$), (2) directed significantly fewer head butts towards larger opponents (GLMs: $P < 0.001$) and (3) were more consistent in the number of head butts aimed at their opponent in relation to their own size compared to control individuals (GLMs: $P \leq 0.002$; Supplementary Fig. S2). Similarly, in same-sex pairings, dsx^{RNAi} males were more consistent in the number of head butts directed at their WT opponent in relation to their own size than were control individuals (GLM: $P = 0.004$; Supplementary Fig. S3). Thus, we found a range of interactions between the effect of *dsx* downregulation and the size of dsx^{RNAi} individuals or their opponent on aggressive behaviours that were shared between male and female dsx^{RNAi} individuals.

We also detected two size-related interactions that were not shared between dsx^{RNAi} males and females. In mixed-sex interactions, larger dsx^{RNAi} males were significantly less likely to approach females and dsx^{RNAi} females were significantly less likely to retreat from larger males (GLM: all $P \leq 0.004$; Supplementary Fig. S4). In both instances, control individuals did not change their behaviours much as a function of either their own body size or that of their opponent.

Interactions Between dsx^{RNAi} *Treatment and Age*

Older dsx^{RNAi} males showed a significantly elevated level of aggression towards females, indicated by a higher number of first head butts and total head butts directed at the females (both GLMs: $P \leq 0.028$), whereas older control males showed reduced aggression towards females (Supplementary Fig. S5a, c). Interestingly, the aggressiveness of dsx^{RNAi} males towards other males remained fairly constant with age, whereas control males became significantly more aggressive with age, indicated by the number of head butts directed at their opponent (GLM: $P = 0.042$; Supplementary Fig. S5b). Thus, for dsx^{RNAi} males, aggression increased with age, but only in mixed-sex interactions.

In dsx^{RNAi} females, increased age resulted in a significant decrease in the tendency to approach their male or female opponent (GLM: all $P \leq 0.005$; Supplementary Fig. S6a, b), in contrast to the pattern found in control females. When paired with males, the likelihood of dsx^{RNAi} females retreating did not change with age, whereas control females were significantly more likely to retreat with age (GLM: $P < 0.001$; Supplementary Fig. S6c). When paired with females, dsx^{RNAi} females were less likely to retreat the older they were, whereas the behaviour of control females remained relatively unaffected by age (GLM: $P = 0.009$; Supplementary Fig. S6d). Overall, for females, dsx^{RNAi} treatment seemingly reduced the willingness to initiate interactions with other male or female beetles with age.

DISCUSSION

In this study, we investigated the role of the transcription factor *dsx* in the regulation and integration of behavioural and morphological phenotypes, with a focus on courtship and aggression in male and female *O. taurus*. As predicted, *dsx* downregulation affected male and female aggressive behaviours, yet we found this effect to be heavily influenced by social context: compared to control individuals, dsx^{RNAi} males were significantly more aggressive in same-sex interactions and significantly less aggressive in intersexual interactions, whereas dsx^{RNAi} females were significantly more aggressive only in intersexual interactions. Unexpectedly, and in contrast to our predictions, *dsx* downregulation increased rather than decreased aggressiveness in hornless dsx^{RNAi} males, while leaving both male and female courtship behaviour unaffected.

dsx *Alters Aggressive Behaviour in a Sex- and Social Context-dependent Manner*

The effect of *dsx* on both male and female aggressive behaviour is noteworthy for several reasons. To the best of our knowledge, this is the first report outside the extensive body of literature on *Drosophila* that documents a significant role of *dsx* in the regulation of male and female behaviour (e.g. Pan & Baker, 2014; Rezával et al., 2012). While *dsx* has previously been shown to regulate sex-specific morphological differentiation across a wide range of insects and noninsect arthropods (reviewed in Price, Egizi, & Fonseca, 2015), our results now raise the possibility that this conservation of function may also apply to the regulation of behaviour. Similarly, while the genetic basis of aggression in insects has received significant prior attention, again the vast majority of research was focused on *D. melanogaster* (e.g. Shorter et al., 2015; Wang, Dankert, Perona, & Anderson, 2008) and *Apis mellifera* (e.g. Alaux et al., 2009; Rittschof & Robinson, 2013). Our present study thus extends this focus beyond established model systems.

At the same time, our results suggest that *dsx* may not only be involved in the development of intersexual behavioural differences per se, but possibly also in the regulation of intrasexual, environment-sensitive behavioural repertoires, such as those distinguishing alternative male morphs. Importantly, our results show that aggressiveness is significantly affected by *dsx* knockdown, thus raising the possibility that *dsx*'s role in behaviour modulation may itself be condition dependent. If correct, these conclusions would parallel those that have emerged from several recent transcriptomic and functional studies in the same species, which document that *dsx* affects sex-specific morphological differentiation via a target gene repertoire that is not only specific for each tissue type and sex, but also modulated heavily by environmental conditions (Kijimoto et al., 2014; Ledón-Rettig & Moczek, 2016; Ledón-Rettig et al., 2017).

Similarly, we find that the behavioural consequences of *dsx* downregulation are conditional upon social context: dsx^{RNAi} males were more aggressive towards other males (intrasexual aggression) and less aggressive towards females, whereas dsx^{RNAi} females only exhibited elevated aggression towards males (intersexual aggression), but not towards other females. Thus, *dsx* does not necessarily affect aggressiveness in males and females systemically and invariably, but rather contributes to a more nuanced and flexible behavioural response. Taken together, the multifaceted and condition-responsive function of *dsx* in the development of behaviour in males and females highlights its potentially integral role in mediating both canalized and plastic behavioural diversity in this and potentially other insect species.

dsx^{RNAi} *Reduces Horns, but Increases Aggressiveness: Revisiting the Fighter–Sneaker Dichotomy*

dsx^{RNAi} drastically reduces nutrition-responsive horn growth in major males (i.e. it converts their morphological appearance towards that of minor (hornless) males; Kijimoto et al., 2012). In this study, we show that the same manipulation increases aggressiveness in these same males. Given that this behavioural phenotype is typically associated with major (horned) males, this increase in aggressiveness was unexpected. Our results suggest that the behavioural categorization of alternative fighter and sneaker wild-type phenotypes in *O. taurus* may be more complex than previously described and therefore may benefit from a critical re-evaluation.

Recall that minor males, like horned major males, engage in head-to-head combat behaviour when first encountering a male opponent, which can lead to prolonged fights with same-sized male opponents. However, in striking contrast to major males,

once defeated, minor males will reattempt to access male-guarded females through the use of a series of alternative behaviours, often over the course of days (Moczek & Emlen, 2000). Our results suggest that when encountering similar-sized males, hornless minor males may interact with their opponent in an unexpectedly aggressive manner. Two nonexclusive explanations may help to interpret this finding. First, since *O. taurus* males gain fertilizations in direct proportion to the number of matings with a female (Hunt & Simmons, 2002; Simmons, Beveridge, & Krauss, 2004), it is conceivable that, in encounters between same-sized minor males, the benefit of aggressively evicting a contender from the tunnel system may exceed that of trying to nonaggressively sneak copulations, which instead is favoured only when engaging larger, physically dominant opponents. If correct, this interpretation suggests that hornless males are capable of displaying high levels of aggression akin to that of horned males yet under a narrower set of circumstances, and that dsx^{RNAi} amplifies this aggressiveness. Alternatively, the behavioural repertoire of wild-type minor males, often described in the literature as simply 'nonaggressive sneaking', may be better described as exhibiting a level of aggressiveness reminiscent of that of major males, as well as behavioural flexibility and especially persistence not seen in their major counterparts. Thus, the increase in interaction time, head butts and first head butts thrown by dsx^{RNAi} males towards their wild-type male opponents in our study might alternatively be interpreted as being reflective of the elevated persistence in engaging opponents over hours, typically seen only in minor males (Moczek & Emlen, 2000). However, further experiments are necessary to disentangle the role of *dsx* in the regulation of the complex behavioural repertoires of *O. taurus* (Kijimoto et al., 2012).

Female Aggressiveness Is Surprisingly High and Affected by dsx

dsx^{RNAi} females were more aggressive towards males, but also retreated more often from males than did control females. This finding parallels results in *D. melanogaster* where insertion-mediated disruption of the *doublesex* locus results in significantly elevated female aggression (i.e. wing flicks, kicking) and rejection behaviours towards males, reducing copulation frequencies, at least at the beginning of sexual interactions (Rideout, Dornan, Neville, Eadie, & Goodwin, 2010).

Overall, females exhibited surprisingly high levels of aggression independent of *dsx*-knockdown (Supplementary Fig. S1). To our knowledge, this is the first study that reports and quantifies female aggression in *Onthophagus*, and hence little prior data exist that could be used to guide the formulation of specific hypotheses. One exception constitutes the documented existence of kleptoparasitism by conspecifics: *O. taurus* females experience brood parasitism from conspecific females (Moczek & Cochrane, 2006) and males (Crowe, Raspet, Rychtar, & Gupta, 2013), and females use the brood balls of other females as food source for their own offspring or themselves (Crowe et al., 2013). Female aggression towards conspecifics could thus be adaptive to prevent or reduce the probability of intraspecific kleptoparasitism. Alternatively, females may use aggression towards males to directly assess male quality related to indirect benefits (e.g. good genes related to strength, vigour, persistence sensu Andersson, 1994; note that female *O. taurus* prefer males with increased courtship rate: Kotiaho, Simmons, & Tomkins, 2001; McCullough & Simmons, 2016) or direct benefits (e.g. levels of disease or parasitism, sensu Andersson, 1994). More generally, the presence of surprisingly high aggression levels among female *O. taurus* adds an important facet to the behavioural phenotypes and mating system of this species, which has mostly been described in the light of male–male competition and associated aggression.

The correlations between morphological and behavioural phenotypes following dsx^{RNAi} were in opposite directions for male and female *O. taurus*: dsx^{RNAi} males showed greatly reduced horn development yet elevated aggressiveness, whereas dsx^{RNAi} females gained both horn development and elevated aggression. These results are especially interesting in the light of the transcriptomic mechanisms of *dsx*-mediated morphological differentiation between the sexes. Ledón-Rettig et al. (2017) showed that the genes responsive to *dsx* knockdown and in possession of putative *dsx*-binding sites in their promotor regions are highly tissue specific and, for all but one tissue analysed, nonoverlapping across sexes. In other words, even in homologous male and female tissues, *dsx* relies on different target gene repertoires to mediate sex-biased differentiation, making it conceivable that the regulation of behaviours via *dsx* may be similarly decoupled not only from that of morphology, but also between the sexes.

In conclusion, our study shows that *dsx* is an important modifier of aggression, affecting both male and female aggressive behaviours in a highly context-dependent manner. These features render this transcription factor as a potent candidate for future studies into the regulation and evolution of canalized and plastic behavioural diversity.

Acknowledgments

We thank Wendy Anderson and Justin Choi for their help with animal care and Thomas Jackson for statistical support. We also thank two anonymous referees for their helpful criticism and editing of the manuscript. This research was supported by the National Institutes of Health to O.M.B. (T32 HD049336-09). Partial support was also provided by National Science Foundation grants to A.P.M. (IOS 0744585, 1120209 and 0820411). The content of this article does not necessarily represent the official views of the National Institutes of Health or the National Science Foundation.

Supplementary material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2017.08.011.

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