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SHORT COMMUNICATION

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A role for sex-determination genes in life history evolution? *Doublesex* mediates sexual size dimorphism in the gazelle dung beetle

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Abstract

An organism's fitness depends strongly on its age and size at maturation. Although the evolutionary forces acting on these critical life history traits have been heavily scrutinized, the developmental mechanisms underpinning intraspecific variation in adult size and development time remain much less well-understood. Using RNA interference, I here show that the highly conserved sex-determination gene *doublesex* (*dsx*) mediates sexual size dimorphism (SSD) in the gazelle dung beetle *Digitonthophagus gazella*. Because *doublesex* undergoes sex-specific splicing and sex-limited isoforms regulate different target genes, this suggests that *dsx* contributes to the resolution of intralocus sexual conflict in body size. However, these results contrast with previous studies demonstrating that *dsx* does not affect body size or SSD in *Drosophila*. This indicates that intraspecific body size variation is underlain by contrasting developmental mechanisms in different insect lineages. Furthermore, although male *D. gazella* have a longer development time than females, sexual bimaturism was not affected by *dsx* expression knockdown. In addition, and in contrast to secondary sexual morphology, *dsx* did not significantly affect nutritional plasticity in life history. Taken together, these findings indicate that *dsx* signalling contributes to intraspecific life history variation but that *dsx's* function in mediating sexual dimorphism in life history differs among traits and species. More generally, these findings suggest that genes ancestrally tasked with sex determination have been co-opted into the developmental regulation of life history traits and may represent an underappreciated mechanism of life history evolution.

KEYWORDS
life history evolution, phenotypic plasticity, RNA interferrence, Scarabaeidae, sexual selection & conflicts

1 | INTRODUCTION

Age and size at maturity are tightly related to an individual's fitness and thus represent key life history components. Large individuals are often more competitive in resource and mate competition, produce more offspring and survive better compared to smaller conspecifics (Blanckenhorn, 2000; Honek, 1993; Peters, 1986). However, growing large also often incurs viability costs related to prolonged development and increased growth (Dmitriew, 2011). The costs and benefits of investment into growth and size are thus heavily context dependent. As males and females often differ in their reproductive interests (most notably due to anisogamy; Bateman, 1948),

sexes commonly differ in optimal trait values (Fairbairn et al., 2007). Consequently, sexual size dimorphism and sexual bimaturism are widespread in anisogamous organisms and contribute greatly to intraspecific variation in nature (Fairbairn, 1997). However, although a large body of literature documents the role of behaviour and sexual as well as ecological selection in driving variation in size, age at maturity, sexual size dimorphism and sexual bimaturism (Badyaev, 2002; Blanckenhorn, 2005; Blanckenhorn et al., 2020; Hirst et al., 2015; Shine, 1989; Stillwell et al., 2010; Temeles et al., 2000), the developmental genetic mechanisms underpinning intraspecific body size variation remain far less well understood (but see e.g.: Millington et al., 2021; Rideout et al., 2016; Rohner et al., 2017; Shingleton, 2011; Stillwell & Davidowitz, 2010). This hampers our understanding of how life histories evolve, how sex differences arise, and whether different lineages depend on similar or divergent mechanisms.

The development of sexual dimorphism in life history traits is of particular interest to evolutionary ecologists because traits such as age and size at maturity are expected to be highly polygenic. As males and females (most often) share almost their entire genome, selection in one sex is expected to cause correlated responses in the other via pleiotropy and/or linkage, thereby generating antagonistic fitness effects and provoking intralocus sexual conflict (Arnqvist & Rowe, 2005; Lande, 1980). Although such conflict can be resolved by relocating genes with sexually antagonistic fitness effects onto sex chromosomes (Dean & Mank, 2014), adjusting the sex-ratio of offspring (Connallon & Jakubowski, 2009), or by silencing maternal or paternal alleles via genomic imprinting (Day & Bonduriansky, 2004; Patten & Haig, 2008), sex-specific regulation of shared autosomal genetic material appears to be the most common mechanism (Ellegren & Parsch, 2007; Grath & Parsch, 2016; Mank, 2017). However, the detailed developmental mechanism underlying sexual dimorphism in life history remain poorly understood.

To further our understanding of life history evolution, I here investigate the developmental mechanisms underpinning sexual size dimorphism and sexual bimaturism in the dung beetle *Digitonthophagus gazella* (Fabricius, 1787). Native to Southern Africa, this scarabaeid has been purposefully introduced as a beneficial species in pasture management in Australia and the Americas (Noriega et al., 2010; Tyndale-Biscoe, 1990). Relative to other members in the tribe Onthophagini, *D. gazella* is a relatively large species, and possesses large intraspecific size variation, with males growing to larger body sizes and developing for longer than females (unpublished). Like closely related species, male *D. gazella* develop a pair of cephalic horns used during male–male combat. Although the developmental mechanisms underlying intraspecific variation in secondary sexual traits have received a lot of attention in dung, rhinoceros and stag beetles (Casasa et al., 2020; Gotoh et al., 2014; Ito et al., 2013; Kijimoto et al., 2012; Zinna et al., 2018), little is known about the developmental regulation of life history traits.

Using functional genetics and nutritional manipulation, I here investigate the functional underpinnings of intraspecific variation in body size and development time—two key life history traits (Roff, 2002; Stearns, 1992). Previous research shows that the transcription factor *doublesex* mediates the development of sex-limited cephalic horns and its nutrition-responsiveness (Moczek & Kijimoto, 2014). Because this gene has also been shown to integrate morphology with behaviour in dung beetles (Beckers et al., 2017), I use a fully factorial design to test whether *dsx* may also contribute to nutritional plasticity and sexual dimorphism in life history. I compare these findings to those made in other species and discuss the implications of the co-option of sex-determination pathway into the developmental evolution of life histories and the resolution of intralocus sexual conflict.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry

Digitonthophagus gazella was collected in Santa Fe, Florida, in spring 2019 and shipped to Bloomington, Indiana, where a laboratory colony was established following standard procedures and kept at constant 29°C.

2.2 | Laboratory rearing and nutritional manipulation

To investigate the developmental underpinnings of variation in body size and development time, larvae were reared under standardized laboratory conditions, crossing a nutritional manipulation with the application of RNA interference. First, 6 females were haphazardly selected from the laboratory colony and transferred into rectangular oviposition containers (27 cm × 17 cm × 28 cm) that were filled with a sterilized sand-soil mixture and topped off with ca. 800 g defrosted cow dung. Reproductively active females dig vertical tunnels (typically 10–30 cm deep) immediately underneath the dung pat and, pulling dung form the surface, construct several compact spheres out of dung in which a single egg is laid. After 5 days, these so-called "brood balls" were sifted from the soil. Because body size is strongly dependent on larval nutrition and maternal investment in this species (Moczek, 1998), offspring were removed from their natal brood balls and placed in standardized, artificial brood balls as described previously (Shafiei et al., 2001). In brief, all natal brood balls were opened and eggs or newly hatched first instar larvae (L1) were transferred into separate wells of a standard 12-well tissue culture plate (as in Rohner & Moczek, 2020).

To manipulate larval nutrition, half or all animals received a full well (3.2 g) of homogenized cow dung, whereas the other half received only 50% as much food (1.6 g). These two treatments are hereafter referred to as high- and low-quality nutrition, respectively. Before the start of the experiment, cow dung was thoroughly mixed using a hand-held electric cement mixer (Nordstrand, PWT-PM0) and several aliquots were frozen and thawed for larval rearing as needed.

2.3 | RNA interference: dsRNA synthesis and injection

To assess the function of *dsx* in the regulation of life history, I applied RNA interference (RNAi). RNAi is a post-transcriptional process triggered by the exposure of an organism to double-stranded RNA (which, in this case, is specific to *dsx*), which leads to systemic gene silencing in a sequence-specific manner (Wilson & Doudna, 2013). That is, RNAi causes quantitative expression knock*down* as opposed to qualitative expression knock*out*. Although the success rate of RNAi is dependent on the nucleotide sequence and the developmental stage, it works reliably for *dsx* in dung beetles (Casasa et al., 2020; Ledón-Rettig et al., 2017). RNAi was applied in half of all individuals within a given 12-well plate following Casasa et al., (2020) including individuals subjected to both nutritional treatments. In brief, *dsx* template DNA was amplified by PCR using *dsx*-specific primers attached to a T7 promoter sequence. MEGAscript T7 transcription and MEGAclear kits (Invitrogen) were used to synthesize and purify dsRNA. The dsRNA was then diluted in injection buffer to reach a concentration of 1.0 μg/μl dsRNA. Using a hand-held syringe, 3 μg dsRNA were consequently injected into the thorax of early L3 larvae. Control injections were performed by injecting buffer solution only. Larvae were inspected daily and the age at pupation, as well as the age at adult emergence was recorded. Pupae were weighed using a Mettler Toledo (AL54 Ohio, USA, $d = 0.1$ mg) scale. After complete sclerotization, emerging adults were sacrificed and stored in 70% ethanol. Of the 144 larvae used in the experiment, 102 survived to the adult stage. Neither treatment significantly affected survival (binomial generalized linear mixed model with plate as random intercept: nutrition: $X^2_{(1)} = 0.01$, $p = .905$; dsx^{RNAi}: $X^2_{(1)} = 2.366$, $p = .124$; Figure S1).

2.4 | Morphometric measurements and statistical analysis

Calibrated pictures of the pronotum, the fore and hind legs, the elytra, as well as the head of each adult individual were obtained using a digital camera (Scion) mounted on a Leica MZ-16 stereomicroscope. Using tpsDig2 (Rohlf, 2009), I then took eight linear measures for pronotum width, pronotum length, elytra length, elytra width, metatibia length, profemur length, profemur width and head width. Because the choice of a body size measure can affect inferences (Fairbairn et al., 2007), three different approaches were used. Firstly, pronotum width was used to estimate overall body size. This is a widely applied linear measure in dung beetles (Emlen, 1994). Secondly, as a more inclusive measure of overall size, I used the cube root of pupal mass. This measure is less dependent on scaling relationships of a specific structure, but is expected to be affected by more sources of variation, such as water content, etc. Lastly, I also used a multivariate approach to estimate body size. To this end, I performed a principal component analysis (based on the covariance matrix of log-transformed values) of all eight linear traits measured and used the scores on the dominant eigenvector as estimates of body size (for more details see: Cheverud, 1982; Klingenberg, 1996). As estimates of development time, the duration of the third (and final) larval instar as well as the duration of the pupal stage were used.

To test for a role in *dsx* in mediating sexual dimorphism and nutritional plasticity in life history, I used mixed models (as implemented in the R-package *lmerTest* (Kuznetsova et al., 2017) with type II sums of squares (using the function *ANOVA*( ) as implemented in the *car* package (Fox & Weisberg, 2019)) to test for the effects of sex, nutritional treatment, dsRNA injection and all interactions on body size and development time. Nonsignificant interactions were removed (unless $p \leq .1$). To account for micro-environmental variation, the 12-well plate an individual was reared in was added as random intercept.

3 | RESULTS AND DISCUSSION

3.1 | *Dsx* mediates sexual size dimorphism

To further our understanding of the developmental regulation of age and size at adult emergence, I here investigate the function of the somatic sex-determination gene *doublesex*. Knocking down *dsx* expression via RNA interference tended to *de*crease male size but *in*creased the size of females (Figure 1a), indicating that *dsx* signalling is required for the development of SSD. Sex × injection − interactions were statistically significant when using pronotum width (Table 1A,

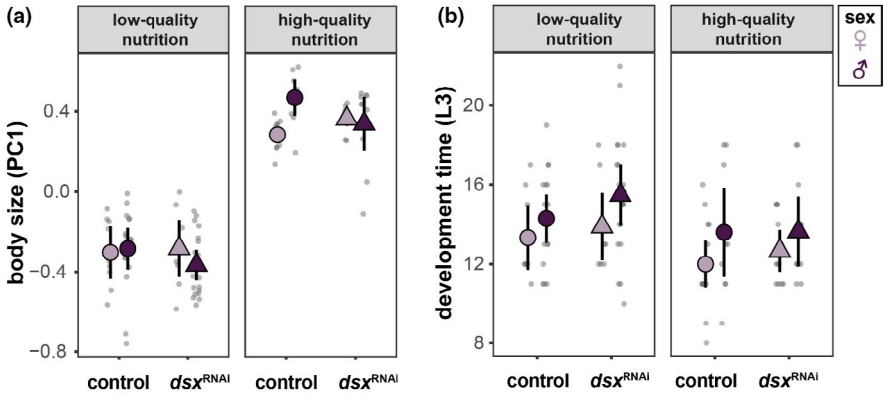

FIGURE 1 Effect of *doublesex* expression knockdown (dsx^{RNAi}) and nutritional quality on body size (a) and development time (b). Error bars indicate standard 95% confidence limits

TABLE 1 Linear mixed model of life history traits as a function of sex, nutritional treatment (high vs. low quality) and injection treatment (3 μl injection buffer solution vs. 3 μg *dsx* dsRNA dissolved in 3 μl injection buffer solution; $n = 102$)

	(A) Log pronotum width					(B) PC1			
	X^2	*df*	*p*	η_p^2		X^2	*df*	*p*	η_p^2
Sex	0.66	1	.418	0.01	Sex	0.61	1	.435	0.01
Nutrition	191.00	1	<.001	0.68	Nutrition	152.42	1	<.001	0.63
Injection	0.15	1	.699	<0.01	Injection	0.71	1	.400	<0.01
Sex × injection	5.71	1	.017	0.06	Sex × injection	11.62	1	.001	0.12
					Sex × nutrition	3.99	1	.046	0.04
	(C) Log pupal weight$^{1/3}$					(D) Development time L3			
	X^2	*df*	*p*	η_p^2		X^2	*df*	*p*	η_p^2
Sex	2.38	1	.123	0.03	Sex	7.77	1	.005	0.08
Nutrition	187.66	1	<.001	0.68	Nutrition	3.00	1	.083	0.03
Injection	0.13	1	.716	<0.01	Injection	2.08	1	.150	0.02
Sex × injection	2.78	1	.095	0.03	Sex × injection	0.30	1	.581	<0.01
	(E) Development time pupa								
	X^2	*df*	*p*	η_p^2					
Sex	<0.00	1	.998	<0.01					
Nutrition	16.50	1	<.001	0.15					
Injection	0.48	1	.489	<0.01					
Sex × injection	1.02	1	.313	0.01					

Note: The 12-well plate an individual was reared in was used as a random effect. Nonsignificant interactions were removed (if $p < .1$) except for the sex × injection − interaction as this was of a priori interest. Partial eta squared (η_p^2) is given as an effect size.

Figure S2a) or a multivariate estimate of size (PC1; Table 1B), but only a trend was present when using pupal mass as size estimate (Table 1C, Figure S2b). These findings for overall size mirror sexually antagonistic effects previously demonstrated for the length of cephalic horns in this and closely related species (Kijimoto et al., 2012), mandibles in stag beetles (Gotoh et al., 2014), as well as butterfly wing development (Iijima et al., 2019), and neuronal development and sexual behaviour in *Drosophila* (Rideout et al., 2010). This suggests that in addition to morphology and behaviour, *doublesex* also contributes to intraspecific variation in life history via its effects on male and female body size and SSD.

Even though the sex-determination pathway upstream of *dsx* is divergent across insect orders, *dsx* structure and function are highly conserved, in particular, the expression of male- and female-specific isoforms generated through alternative splicing of an exon that is present in male *dsx* transcripts but absent in those expressed in females (Shukla & Nagaraju, 2010; Wexler et al., 2019). Sex-specific isoforms differ in the identity of target genes as well as the direction in which target gene expression is modified (Ledón-Rettig et al., 2017). *Dsx*, therefore, acts as a developmental switch that uncouples gene expression in one sex from that in the other. Hence, this mechanism has the potential to effectively resolve intralocus sexual conflict and mediate sex-specific development. Although sex-specific fitness functions for *D. gazella* are currently lacking, size is linked to fecundity and reproductive success in closely related dung beetle species (e.g. Hunt & Simmons, 2002), and body size is often thought to be subject to sexually antagonistic selection (Fairbairn et al., 2007). The co-option of *dsx* in the regulation of SSD may thus serve as a simple mechanism able to resolve intralocus conflict in life history as well.

Interestingly, *dsx* does *not* affect SSD in *Drosophila melanogaster* (Hildreth, 1965; Rideout et al., 2016). In this species, female-biased SSD is driven by the female-limited expression of *transformer*, but independent of *doublesex* (Rideout et al., 2016). This suggests that *dsx's* role in life history can evolve and, given the ubiquity of *dsx* signalling in hexapods (Price et al., 2015; Verhulst & van de Zande, 2015), may represent an underappreciated regulator of life history variation in insects.

3.2 | Sexual bimaturism is independent of *dsx*

In addition to body size, I also tested whether *dsx* affects development time and sexual bimaturism. Larvae reared on low-quality nutrition tended to take longer to reach the pupal stage (Figure 1b, Table 1D) yet had accelerated pupal development (Table 1E, Figure S2c). Although males spent more time in the third larval instar compared to females (sex main effect: $X^2_{(1)} = 7.77$, $p = .005$), sexual bimaturism was not affected by dsx^{RNAi} (sex × injection − interaction:

$X^2_{(1)} = 0.30$, $p = .581$). This suggests that not all sex differences in life history are linked to *dsx*, or at least not to the same extent, implying that other regulators of sexual dimorphism in life history remain to be identified.

3.3 | *Dsx* does not mediate nutritional plasticity in life history

Previous work shows that *dsx* not only mediates sexual dimorphism but also nutritional plasticity in secondary sexual traits (Casasa et al., 2020; Gotoh et al., 2014; Rohner et al., 2021). I found that body size increased with nutritional quality (log pronotum width: $X^2_{(1)} = 191.00$, $p < .001$; log pupal weight$^{1/3}$: $X^2_{(1)} = 191.00$, $p < .001$; PC1: $X^2_{(1)} = 152.42$, $p < .001$). However, in contrast to the sex-limited cephalic horns in this and other species (Moczek & Kijimoto, 2014), *dsx* knockdown did not affect (sex-specific) nutritional plasticity of body size (Table 1). Note, however, that sexual size dimorphism was more male-biased in control individuals that were exposed to high-quality nutrition. This is in agreement with other studies demonstrating that SSD increases with nutritional quality (Rohner et al., 2017, 2018; Teder & Tammaru, 2005). Yet, sex-specific plasticity was only significant when using PC1 as a size estimate and was not affected by *dsx* knockdown (nonsignificant sex × nutrition × injection-interaction). This suggests that *dsx* mainly affects body size in a sex-specific but largely nutrition-independent manner.

4 | CONCLUSIONS

Using functional genetic manipulations in a standardized laboratory setting, I here show that the somatic sex-determination gene *doublesex* functions in the regulation of intraspecific variation in body size but not development time. As the sex-specific effects of *dsx* are mediated via sex-limited splice variants (Verhulst & van de Zande, 2015), alternative splicing may represent a currently underappreciated mechanism in the evolution of SSD and life history more generally. Together with previous findings, *dsx* emerges as a potential developmental integrator of hexapod morphology, behaviour, as well as life history. Future research will be necessary to evaluate whether *dsx* contributes to population differentiation and macroevolutionary divergence in body size.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

All data and code used in this study are submitted as supplementary material and available on Dryad (https://doi.org/10.5061/dryad.prr4xgxmd).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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