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SHORT COMMUNICATION

Assessing the evolutionary lability of insulin signalling in the regulation of nutritional plasticity across traits and species of horned dung beetles

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Abstract

Nutrition-dependent growth of sexual traits is a major contributor to phenotypic diversity, and a large body of research documents insulin signalling as a major regulator of nutritional plasticity. However, findings across studies raise the possibility that the role of individual components within the insulin signalling pathway diverges in function among traits and taxa. Here, we use RNAi-mediated transcript depletion in the gazelle dung beetle to investigate the functions of *forkhead box O* (*Foxo*) and two paralogs of the insulin receptor (*InR1* and *InR2*) in shaping nutritional plasticity in polyphenic male head horns, exaggerated fore legs, and weakly nutrition-responsive genitalia. Our functional genetic manipulations led to three main findings: $Foxo^{RNAi}$ reduced the length of exaggerated head horns in large males, while neither *InR1* nor *InR2* knock-downs resulted in measurable horn phenotypes. These results are similar to those documented previously for another dung beetle (*Onthophagus taurus*), but in stark contrast to findings in rhinoceros beetles. Secondly, knockdown of *Foxo*, *InR1*, and *InR2* led to an increase in the intercept or slope of the scaling relationship of genitalia size. These findings are in contrast even to results documented previously for *O. taurus*. Lastly, while $Foxo^{RNAi}$ reduces male forelegs in *D. gazella* and *O. taurus*, the effects of *InR1* and *InR2* knockdowns diverged across dung beetle species. Our results add to the growing body of literature indicating that despite insulin signalling's conserved role as a regulator of nutritional plasticity, the functions of its components may diversify among traits and species, potentially fuelling the evolution of scaling relationships.

KEYWORDS
allometry, *Digitonthophagus gazella*, nutritional plasticity, polyphenism, RNA interference, sex-specific plasticity

1 | INTRODUCTION

Nutritional plasticity in the shape and size of morphological traits are major contributors to phenotypic variation, and its evolution is a major source of adaptive diversification, especially for secondary sexual traits (Gould, 1966; Lupold et al., 2016; Rohner & Blanckenhorn, 2018; Thompson, 1917). Traits that function in mate competition, such as ornaments and armaments, are thought to be particularly dependent on their bearer's nutritional status (or individual condition), such that the degree of trait exaggeration serves as an indicator of quality or competitive ability (Andersson, 1994; Emlen et al., 2012; Rowe & Houle, 1996). Given the importance of nutrition-dependent trait development in sexual signalling and trait diversification, the proximate mechanisms that mediate plastic responses to nutrition have received substantial attention in evolutionary developmental biology. One of the major pathways involved in the developmental regulation of nutritional plasticity across sexes and traits is the insulin/insulin-like growth factor signalling (IIS) pathway (Casasa & Moczek, 2018; Green & Extavour, 2014; Mirth & Riddiford, 2007; Vitali et al., 2018). The IIS pathway transduces nutritional variation in the concentration and composition of circulating insulin-like peptides (ILPs) into tissue-specific growth responses. Because insulin-like peptides reflect nutritional status (or 'condition' sensu Rowe & Houle, 1996), the IIS pathway is an intuitive candidate underpinning the evolution and plasticity of secondary sexual traits. This is because, in linking trait expression to an organism's nutritional status (or 'condition'), it renders sexual signals honest (Emlen et al., 2012). However, accumulating evidence suggests that the proximate underpinnings of nutrition-dependent trait expression may be more varied than previously assumed (Casasa & Moczek, 2018). We here investigate the function of three IIS pathway members in regulating plasticity across traits and species of dung beetles.

The role of the IIS pathway in the developmental regulation of morphological traits is best understood in insects (although the pathway itself exists in all metazoans; Skorokhod et al., 1999). Emlen et al. (2012) showed that trait differences in the strength of nutritional plasticity among strongly nutrition-responsive male horns, less responsive wings, and robust genitalia of Rhinoceros beetles are mediated by the insulin receptor *InR*. Even though this work did not distinguish between the two paralogs *InR1* and *InR2* commonly found in insects, it nevertheless motivated the hypothesis that insulin receptors may constitute a key contributor to the evolution and development of sexual signals. In essence, being at the nexus between systemic signals of nutritional status (ILPs) and tissue-specific growth, *InR* is predicted to facilitate the evolution of weapons and ornaments by rendering their exaggeration an honest signal of individual quality. However, more recent work indicates that the precise mechanisms by which IIS shapes nutrition-responsive growth may vary across systems. For instance, studying the strong nutritional response in head horn length in the horned dung beetle *Onthophagus taurus*, Casasa and Moczek (2018) showed that neither *InR1* nor *InR2* mediate horn length plasticity. Instead, *Foxo*, a transcription factor canonically regarded as a growth repressor (as well as a possible sensitizer of *InR* function; Puig et al., 2003), controlled horn allometry by promoting horn growth in high-nutrition males but reducing it in low-nutrition males. This indicates that different components of the IIS pathway may be recruited in different evolutionary contexts. In addition, even in cases where *InR* regulates nutrition-responsive development, the precise function may be more complex. Xu et al. (2015) showed that the two *InR* paralogues in the planthopper *Nilaparvata lugens* act in an antagonistic manner to determine polyphenic wing development. Complementary findings in other taxa have since demonstrated that the two *InR* paralogues may rather commonly acquire divergent functions (e.g., Okada et al., 2019; Sang et al., 2016; Smykal et al., 2020; Xue et al., 2021), potentially facilitating the evolution of polyphenic development (Xu & Zhang, 2017). However, it remains unclear whether such divergences occur only over vast phylogenetic distances or may also contribute to differentiation among more closely related taxa. The degree to which the evolution of gene function within the IIS signalling pathway facilitates or constrains diversification in nutritional plasticity thus remains unclear.

Here, we study the role of *Foxo* and *InR* in the scaling of primary and secondary sexual traits in the gazelle dung beetle *Digitonthophagus gazella* (Fabricius, 1787) (see Figure 1). Similar to many other species in the tribe Onthophagini, male *D. gazella* develop a pair of cephalic horns used during male-male combat. These horns are strongly nutritionally plastic, which manifests in a nutritional polyphenism separating small 'minor' males from large horned 'major' males (Casasa et al., 2020; Rohner & Moczek, 2023). In addition, males of this species develop exaggerated and strongly nutrition-dependent forelegs used during mating (Rohner et al., 2021). Taking a functional genetic approach, we assess whether RNAi-mediated gene expression knockdown of *Foxo* and both known paralogs of *InR* affect the nutritional scaling of horns, forelegs, and male genitalia and contrast our findings to those in the relatively closely related *Onthophagus taurus* (divergence ~38 mya; Breeschoten et al., 2016) and more distantly related Rhinoceros beetle *Trypoxylus dichotomus* (~140 mya; McKenna et al., 2019). Our results highlight that even though the insulin signalling pathway consistently emerges as a major mediator of nutritional plasticity, the precise functions of pathway components may diverge among traits and species.

2 | MATERIALS AND METHODS

2.1 | Laboratory rearing and nutritional manipulation

Digitonthophagus gazella was collected in Santa Fe, Florida, in spring 2019 and shipped to Bloomington, Indiana, United States, where a laboratory colony was established following standard procedures and kept at a constant 29°C. To generate larvae for dsRNA and buffer injections, we transferred adult females into rectangular oviposition containers (27 cm × 17 cm × 28 cm) filled with a sterilized sand-soil mixture and defrosted cow dung. Females

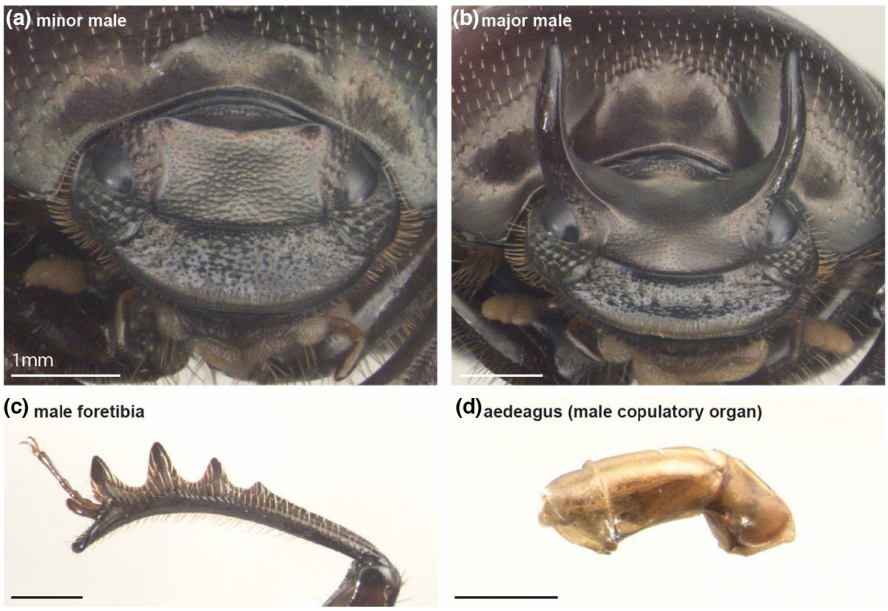

FIGURE 1 The head morphology of *Digitonthophagus gazella* is sexually dimorphic and strongly nutrition dependent in males. Small males (a) develop minute horns, while large males (b) develop large, curved cephalic horns. Males develop nutritionally plastic elongated fore tibiae (c) used during mating. In contrast to head horns and tibiae, the size of the male copulatory organ (i.e., the aedeagus; panel d) is relatively nutrition-insensitive. Scale bar represents 1 mm.

were allowed to produce brood chambers (so-called brood balls) in the soil. After 5 days, all brood balls were collected. Offspring were extracted from their natal brood balls and placed in standardized artificial brood balls as described previously (e.g., Shafiei et al., 2001). In brief, we opened all natal brood balls and transferred eggs or newly hatched larvae into separate wells of standard 12-well tissue culture plates. To ensure larvae experienced a range of nutritional qualities, we provisioned larvae with a mix of dung of cows that were fed with grass or hay. Such differences in bovine diet increase the variation in nutritional quality and adult body size (Rohner & Moczek, 2021) and facilitate the detection of functional genetic effects on nutritional plasticity.

2.2 | RNA interference: dsRNA synthesis and injection

To assess the function of *Foxo*, *InR1*, and *InR2* in the regulation of nutritional scaling, we applied RNAi-mediated gene expression knockdown in half of all individuals following Casasa and Moczek (2018) (also see: Snell-Rood & Moczek, 2012). In brief, we obtained 250 bp DNA constructs for each gene (Integrated DNA Technology, IDT) containing the genomic sequences of *Foxo*, *InR1*, and *InR2* in *D. gazella* (Table S1). Constructs were amplified by PCR using gene-specific primers attached to a T7 promoter sequence. MEGAscript T7 transcription and MEGAclear kits (Invitrogen) were used to synthesize and purify dsRNA. Double-stranded RNA was then diluted in injection buffer to reach a concentration of 1.0 μg/μL dsRNA. Using a hand-held syringe, 3 μg of dsRNA were subsequently injected into the thorax of early third-instar larvae. Control injections were performed by injecting buffer solution only. Mortality after injections was generally low and did not differ strongly among treatments (buffer: 7.2% [$n = 69$]; $Foxo^{RNAi}$: 12.2% [$n = 74$]; $InR1^{RNAi}$: 8.0% [$n = 50$]; $InR2^{RNAi}$: 6.3% [$n = 48$]). After complete sclerotization, emerging adults were sacrificed and stored in 70% ethanol.

2.3 | Morphometric measurements and statistical analysis

We obtained calibrated pictures of the pronotum, the fore tibia, the male head horns, and the male aedeagus using a digital camera (Scion, Frederick, MD, USA) mounted on a Leica MZ-16 stereomicroscope (Bannockburn, IL, USA). Using tpsDig2 (Rohlf, 2009), we then took linear measures to quantify the size of all traits and used pronotum width as an index of body size (see Rohner, 2021). Measurements taken are indicated in Figure S1. To contrast the effects on tibia length in *D. gazella* to *O. taurus*, we revisited the animals generated by Casasa and Moczek (2018) and measured their forelegs (which had not previously been studied).

To test for the effect of RNAi on horn length, we used a 4-parameter log-logistic model including either a common allometric relationship between horn length and body size or two separate curves for each treatment (buffer vs. dsRNA injection). The better-fitting model was selected based on Akaike's Information Criterion (AIC). The effect of RNAi treatment (buffer vs. dsRNA injection) on logarithmized male genitalia and tibia size was tested using linear models. Pronotum width and its interaction with the RNAi treatment were included to account for allometric variation and to test for the effects of gene expression knockdown on allometric scaling. Non-significant interactions were removed.

3 | RESULTS AND DISCUSSION

3.1 | *Foxo*: A potentially conserved mechanism contributing to polyphenic horn development

To investigate whether the functions of different components of the insulin signalling pathway diverge across species and traits, we applied RNAi-mediated gene expression knockdown. Horn length, a nutrition-sensitive threshold trait, was strongly

affected by *Foxo* knockdown (a model including separate scaling relationships per treatment had a lower AIC [−39.64] compared to a model assuming a common allometric relationship [AIC = −21.74]). Specifically, $Foxo^{RNAi}$ decreased horn length in large males while slightly increasing horn length in small males (Figure 2). *Foxo* thus seems to act as a growth inhibitor in small males and a growth promoter in large individuals. These antagonistic effects in major and minor males effectively weaken (or linearize) the sigmoidal relationship, similar to what was previously found in *O. taurus* by Casasa and Moczek (2018). As the presence of cephalic horns as well as their polyphenic expression in *D. gazella* and *O. taurus* represent synapomorphies (Emlen, Hunt, & Simmons, 2005; Emlen, Marangelo, Ball, & Cunningham, 2005), *Foxo*'s conserved function in mediating the developmental switch between minor and major male morphologies may thus be conserved across a vast number of species of horned dung beetles. However, whether *Foxo* functions in the regulation of horn formation in lineages that likely evolved horns independently remains to be investigated. Rhinoceros beetles (subfamily Dynastinae) are known for their greatly exaggerated heads and thoracic horns in males, yet these horns are generally not expressed in a polyphenic manner, and thus *Foxo*'s function,

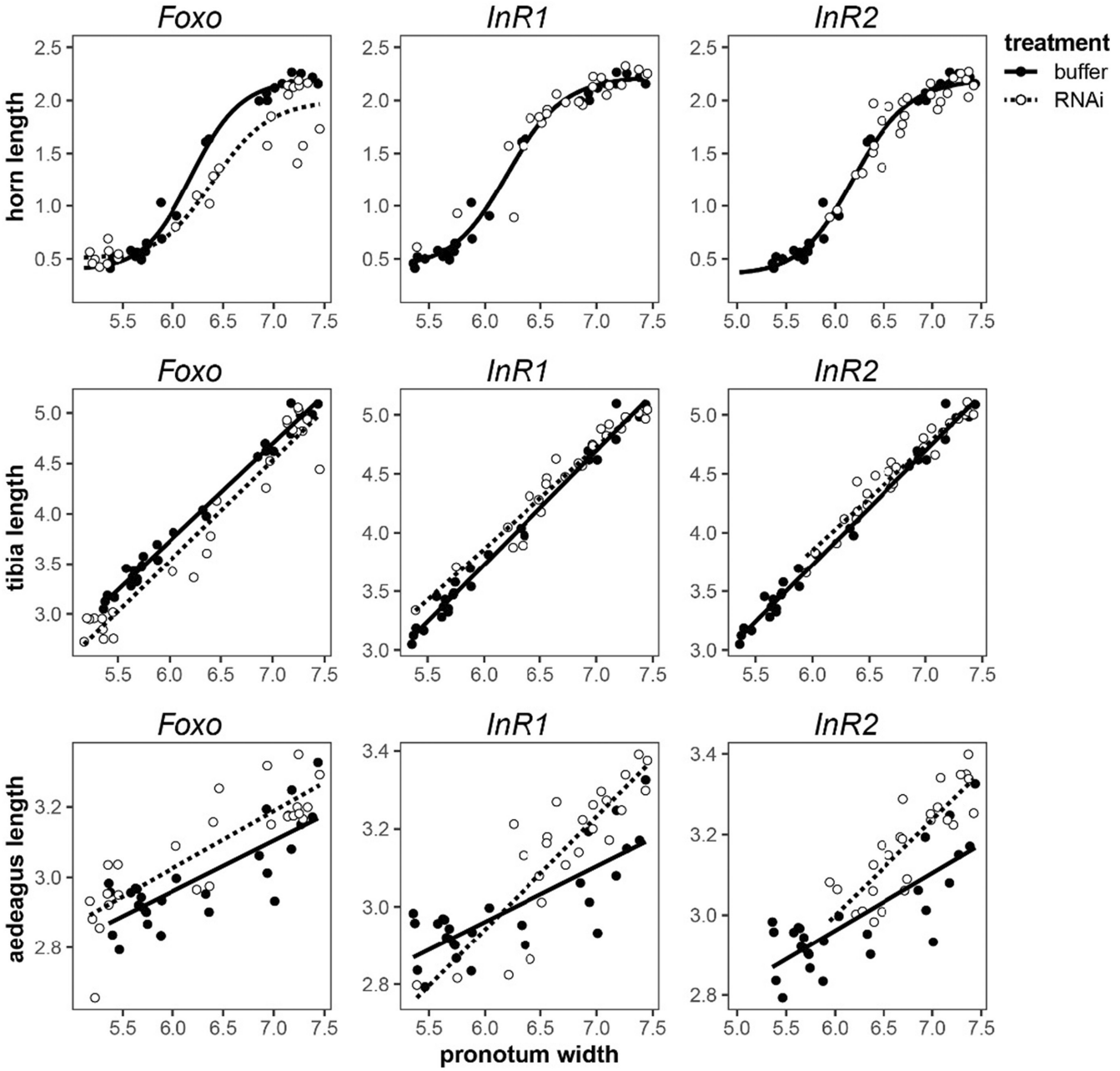

FIGURE 2 Effects of *Foxo*, *InR1*, and *InR2* expression knockdown on horn, tibia, and aedeagus length in *Digitonthophagus gazella*. The sigmoidal scaling relationship of head horns was fitted using 4-parameter log-logistic curves. The scaling of tibia and aedeagus length was modelled using ordinary linear models.

if any, may be rather divergent from that observed in horned dung beetles.

3.2 | Divergence in *InR* function among subfamilies but not members of the same tribe

The insulin receptor has been shown to underpin head horn plasticity in the Rhinoceros beetle (Emlen et al., 2012), but neither paralogue of *InR* affected horn allometry in the dung beetle *O. taurus* (Casasa & Moczek, 2018). In *D. gazella*, we similarly find no evidence for a role of *InR1/2* in the regulation of horn length (Figure 2; AICs were higher in models that include treatment-specific scaling relationships; see Table S1). Together with our *Foxo* results presented above, this suggests that at least part of the developmental architecture underlying nutrition-responsive horn growth may be conserved among species of the same tribe (*Onthophagus* and *Digitonthophagus* diverged ~38 mya; Breeschoten et al., 2016), but diverge across broader phylogenetic scales (Scarabeinae and Dynastinae diverged ~140 mya; McKenna et al., 2019). Note that, similar to findings in Casasa and Moczek (2018), $InR1/2^{RNAi}$ reliably affected aedeagus and tibial development (see below), that is, the absence of horn phenotypes is unlikely to be due to low penetrance. The two *InR* paralogues thus do not seem to mediate polyphenic horn development in either dung beetle species studied so far.

3.3 | Nutrition-dependent development of exaggerated forelegs

D. gazella males develop strongly elongated and hyperallometric forelegs used in mating. We found that $Foxo^{RNAi}$ reduces relative tibia length without affecting the slope of the scaling relationship (Table 1, Figure 2). These effects are similar in direction as in *O. taurus* (see Figure 3 and Table S2), again consistent with a conservation of *Foxo* function among horned dung beetle species. The effects of *InR*, in contrast, differed across species. Knockdown of *InR1* and *InR2 decreased* tibia length in *O. taurus* (Figure 3), consistent with *InR* functioning as a growth promoter. However, knockdown of the same gene in *D. gazella* led to an *increase* in average tibia size (Figure 2). Although modest, these qualitative differences were unexpected and add to the growing number of studies documenting substantial evolutionary lability in the functions of IIS signalling components. Further research will be necessary to determine the representative nature of these results and to assess their molecular genetic underpinnings.

3.4 | Differences in the regulation of genitalia size across species

Genitalia are often thought of as particularly nutrition-*in*sensitive due to presumed stabilizing selection favouring a size that fits

TABLE 1 ANOVA tables (type II Sums of Squares) for the effect of RNAi treatment (buffer vs. dsRNA injection) on logarithmized male tibia and genitalia size for *D. gazella*. Pronotum width and its interaction with the RNAi treatment were included to account for allometric variation and to test for the effects of gene expression knockdown on allometric scaling. Non-significant interactions were removed.

	Foxo				InR1				InR2			
	SS	df	F	p	SS	df	F	p	SS	df	F	p
Log genitalia size												
log pronotum width	0.080	1	99.10	**<0.001**	0.074	1	91.10	**<0.001**	0.053	1	86.50	**<0.001**
Treatment (buffer vs. dsRNA)	0.007	1	8.80	**0.005**	0.004	1	4.90	**0.032**	0.010	1	16.40	**<0.001**
log pronotum width × treatment					0.010	1	12.80	**0.001**	0.003	1	5.70	**0.021**
Residuals	0.040	49			0.038	47			0.030	49		
Log tibia size												
log pronotum width	2.050	1	1234.50	**<0.001**	1.031	1	1932.68	**<0.001**	0.973	1	2106.29	**<0.001**
Treatment (buffer vs. dsRNA)	0.040	1	24.08	**<0.001**	0.005	1	8.71	**0.005**	0.004	1	8.73	**0.005**
log pronotum width × treatment					0.004	1	7.88	**0.007**	0.003	1	5.54	**0.023**
Residuals	0.081	49			0.025	47			0.023	49		

Note: Bold values indicate *p* values smaller than 0.05.

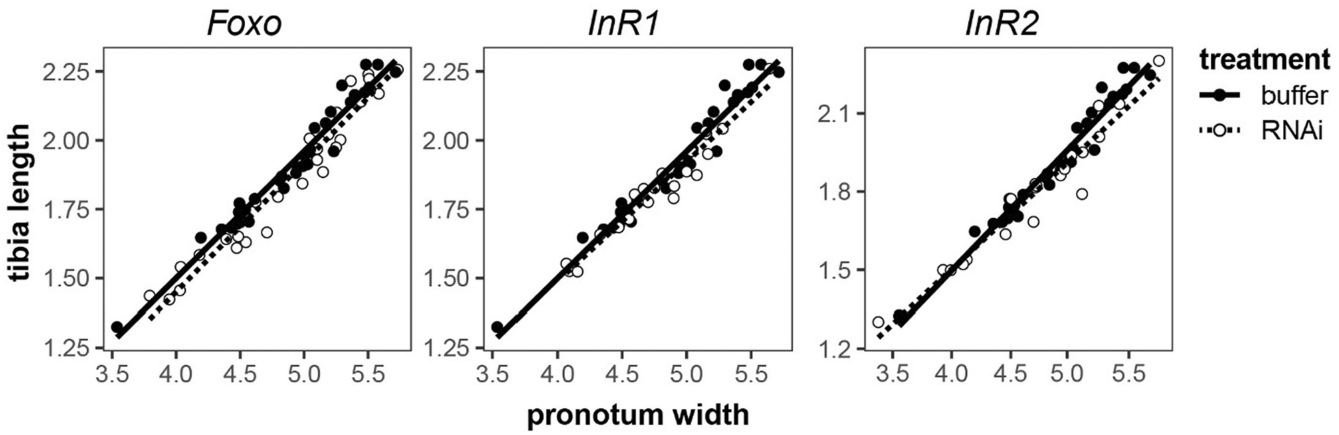

FIGURE 3 Effects of *Foxo*, *InR1*, and *InR2* knockdown on tibia length in *O. taurus*. Animals were generated by Casasa and Moczek (2018) but remeasured to compare gene expression knockdown effects on tibia length between *O. taurus* and *D. gazella*.

most potential partners (e.g., the one-size-fits-all hypothesis; Eberhard et al., 1998). However, the mechanisms allowing genitalia to be robust against nutritional variation are relatively poorly understood. In *Drosophila*, the size of genitalia is less sensitive to nutrition than other traits because *Foxo*, normally expressed to limit organ growth at low nutrition only, is suppressed in the genitalia of low nutrition flies, thereby allowing genitalia to 'ignore' low nutrition conditions (Tang et al., 2011). Thus, $Foxo^{RNAi}$ has little effect on genitalic growth in flies (Tang et al., 2011). In contrast, in *O. taurus*, $Foxo^{RNAi}$ decreased the slope of the genitalia-body size allometry, whereas in *D. gazella*, we find no effect on the slope but an increase in the intercept (Table 1). This suggests that, in contrast to *Drosophila*, $Foxo^{RNAi}$ does influence aspects of genitalic growth in dung beetles, yet the two dung beetle species differ in whether *Foxo* shapes nutrition responsiveness (RNAi effect on slope in *O. taurus*) or average size independent of nutrition (RNAi-mediated shift in intercept in *D. gazella*). Similar differences between the two dung beetles were found for the two *InR* paralogues, where $InR1/2^{RNAi}$ increased the allometric slope of genitalia in *D. gazella* (see Table 1) while decreasing genitalia size independent of body size in *O. taurus* (Casasa & Moczek, 2018). These findings suggest that while the role of some IIS components in shaping plasticity is conserved in some traits (e.g., *Foxo*'s role in horn development), others readily diverge in their trait-specific functions (*Foxo* and *InR1/2* in genitalia). The precise mechanisms and interactions mediating these differences will require further investigation. For instance, insects generally produce several insulin-like peptides that serve as upstream inputs of nutritional information (Bland, 2023; Semaniuk et al., 2021). Future research will be necessary to test whether the evolutionary lability of IIS is driven by the subfunctionalization of specific ILPs (such as in the broad-horned flour beetle (Okada et al., 2019)). Similarly, the degree to which interactions between FOXO and other pathways, such as Fat/Hippo, TOR, or Doublesex (Casasa & Moczek, 2018; Gotoh et al., 2015; Koyama et al., 2013), add to the evolutionary lability of IIS in secondary sexual trait development remains to be investigated.

3.5 | General role of nutritional plasticity in the evolution of secondary sexual traits

The dependence of trait exaggeration on IIS, and *InR* in particular, is often thought to provide a mechanism of honest signalling. That is, while large, well-fed individuals experience high levels of ILPs and strong plastic growth responses (mediated through *InR*), the same degree of exaggeration is constrained in individuals experiencing low-nutrition conditions. If so, secondary sexual trait expression is effectively constrained by organismal condition, which then provides an honest indicator of quality (Emlen et al., 2012; Penn & Szamado, 2020). This is an intuitive hypothesis, especially given the widespread involvement of IIS in trait exaggeration and nutritional plasticity. However, at least in onthophagine dung beetle horns, the mechanisms of trait exaggeration may be more complex. Functional genetic manipulations of the Hedgehog pathway demonstrate that rather than not being able to form exaggerated horns, small males are actively inhibiting their formation (Kijimoto & Moczek, 2016). Similarly, the location of the horn threshold is environmentally plastic and harbours genetic variation (Macagno et al., 2021; Rohner & Moczek, 2023). Small males thus have, in principle, the capacity to develop longer horns, but (status-dependent) selection (Gross, 1996) is likely to favour hornlessness below a critical body size threshold (Hunt & Simmons, 2001). Although horn morphology is linked to the IIS and therefore nutrition, it is more likely that nutritional status serves as a cue for, rather than constraint on, horn development. While this might be a particularity of threshold traits that are associated with alternative reproductive tactics and context-dependent selection, this also suggests that the mere involvement of IIS in the regulation of secondary sexual traits does not require traits to be truly honest signals of their bearer's nutritional status.

4 | CONCLUSIONS

Nutritional plasticity and its evolution constitute a major axis of morphological variation, but the mechanisms underlying its

diversification are more varied than previously assumed. We find that the function of different IIS components evolves across traits and species in horned beetles, albeit to different degrees for different components. These findings document that the mechanisms underpinning condition-dependent trait expression evolve even across relatively closely related species, highlighting underappreciated functional diversity in well-known regulators of nutritional responses. This evolutionary lability may facilitate the trait- and taxon-specific evolution of secondary sexual trait exaggeration.

AUTHOR CONTRIBUTIONS

Sofia Casasa: Conceptualization (equal); methodology (equal); writing – review and editing (equal). **Armin P. Moczek:** Conceptualization (supporting); funding acquisition (lead); investigation (equal); methodology (equal); project administration (equal); writing – original draft (equal); writing – review and editing (equal). **Patrick T. Rohner:** Conceptualization (lead); formal analysis (supporting); funding acquisition (lead); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

All data underlying this study are available on Dryad (doi: 10.5061/dryad.s7h44j1cj).

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jeb.14240.

CONFLICT OF INTEREST STATEMENT

The Authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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